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Translational Research in GastroIntestinal Disorders (TARGID) – IBD group

MOLECULAR CHARACTERIZATION OF PATHOPHYSIOLOGIC PATHWAYS IN IBD AND ITS THERAPEUTIC POTENTIAL

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Contents

LIST OF ABBREVIATIONS

AIM2	Absent in melanoma 2
AMPK	AMP-activated protein kinase
ASC	Apoptosis-associated speck-like protein containing a CARD
ASO	Antisense oligonucleotide
ATG14	Autophagy Related 14
ATG16L1	Autophagy Related 16 Like 1
ATG4D	Autophagy Related 4D Cysteine Peptidase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BF	Brightfield
BiP	Binding immunoglobulin protein
BSA	Bovine serum albumin
CALR	Calreticulin
CARD	Caspase recruitment domain
CARD15	Caspase recruitment domain-containing protein 15
CASP1	Caspase-1
CCS	Complete chelating solution
CD	Crohn's disease
CHMP2A	Charged Multivesicular Body Protein 2A
CHMP4B	Charged Multivesicular Body Protein 4B
CK-18	Cytokeratin-18
CK-20	Cytokeratin-20
DAI	Disease activity index
DAMP	Danger associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAJB11	DnaJ Heat Shock Protein Family (Hsp40) Member B11
DNAJB9	DnaJ Heat Shock Protein Family (Hsp40) Member B9
dsDNA	Double stranded DNA
DSS	Dextran Sodium Sulfate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme linked immunosorbent assay
EMA	European Medicines Agency
eQTL	Expression quantitative trait loci
ER	Endoplasmic reticulum
ERAD	ER-associated degradation

ERC	European Research Council
ESCRT	Endosomal Sorting Complex Required for Transport
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FDR	False discovery rate
FELASA	Federation for Laboratory Animal Science Associations
FHC	Fetal human colon
FKBP14	FK506 Binding Protein 14
GABARAPL1	GABA Type A Receptor Associated Protein Like 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRP78	78 kDa glucose-regulated protein
GvHD	Graft-versus-host disease
HERPUD1	Homocysteine Inducible ER Protein With Ubiquitin Like Domain 1
HIN	Hematopoietic interferon-inducible nuclear protein
HLA	Human Leukocyte Antigen
HMGB1	High mobility group box-1
hpi	Hours post isolation
HRP	Horseradish peroxidase
HSP90B1	Heat Shock Protein 90 Beta Family Member 1
HSPA5	Heat Shock Protein Family A (Hsp70) Member 5
HYOU1	Hypoxia Up-Regulated 1
IBD	Inflammatory bowel disease
IC_{50}	Half maximal inhibitory concentration
IEC	Intestinal epithelial cell
IFI16	Interferon inducible 16
IFIX	Interferon-inducible protein X
IFN	Interferon
IFX	Infliximab
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
i.p.	intraperitoneal
IQR	Interquertile range
IRGM	Immunity Related GTPase M
i.v.	Intravenous
JAK	Janus kinase
JAKINIB	Janus kinase inhibitor
KDELR3	KDEL (Lys-Asp-Glu-Leu) Endoplasmic Reticulum Protein Retention Receptor 3
KI67	Antigen Ki-67/Marker Of Proliferation Ki-67
LGR5	Leucine Rich Repeat Containing G Protein-Coupled Receptor 5

LIMMA	linear models for microarray data
LP	Lamina propria
LPS	Lipopolysaccharide
LRRK2	Leucine Rich Repeat Kinase 2
MADCAM	Mucosal Addressin Cell Adhesion Molecule
MAP1LC3A	Microtubule Associated Protein 1 Light Chain 3 Alpha
MIAME	Minimum information about a microarray experiment
MNDA	Myeloid Cell Nuclear Differentiation Antigen
mNoggin	Murine Noggin
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MTMR3	Myotubularin Related Protein 3
mTOR	Mechanistic/Mammalian target of rapamycin
MTX	Methotrexate
MUC2	Mucin 2
NFAT1	Nuclear factor of activated T-cells
NLR	NOD like receptor
NLRC4	NLR family, CARD domain containing 4
NLRP	NLR family, pyrin domain containing
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
ORMDL3	ORMDL Sphingolipid Biosynthesis Regulator 3
P62	Sequestosome-1
PAMP	Patogen associated molecular pattern
PBA	Phenylbutyrate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCSK9	Proprotein Convertase Subtilisin/Kexin Type 9
PDGFR-α	Platelet derived growth factor receptor-alpha
PDIA5	Protein Disulfide Isomerase Family A Member 5
PDIA6	Protein Disulfide Isomerase Family A Member 6
PE	Phosphatidylethanolamine
PFA	Paraformaldehyde
PRKAB2	Protein Kinase AMP-Activated Non-Catalytic Subunit Beta 2
PRR	Pattern recognition receptor
PtdIns	Phosphatidylinositol
PtdIns3P	PtdIns 3-phosphate
PYHIN	Pyrin and HIN domain family
qRT-PCR	Quantitative realtime-polymerase chain reaction
RA	Risk allele
RA	Rheumatoid arthritis
RIPA	Radioimmunoprecipitation assay

ROCK	Rho-Associated Protein Kinase
ROS	Reactive oxygen species
Rspo1	R-Spondin 1
s.c.	Subcutaneous
SCID	Severe combined immunodeficiency
SEC61A1	Sec61 Translocon Alpha 1 Subunit
SMAD	SMAD family member 3
Smad7	Mothers against decapentaplegic homolog 7
SNP	Single nucleotide polymorphism
SPF	Specific-pathogen-free
SRPR	SRP Receptor Alpha Subunit
SSR1	Signal Sequence Receptor Subunit 1
STAT	Signal transducer, and activator of transcription
SYVN1	Synoviolin 1
TCR	T cell receptor
TDT	Terminal deoxynucleotidyl transferase
Tg	Thapsigargin
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
TPMT	Thiopurine methyltransferase
T_{reg}	Regulatory T cell
TUDCA	Tauroursodeoxycholic acid
TYK2	Tyrosine Kinase 2
UC	Ulcerative colitis
ULK1	Unc-51 Like Autophagy Activating Kinase 1
UPR	Unfolded protein response
USA	United States of America
VCAM	Vascular Cell Adhesion Molecule
WB	Western blot
WFS1	Wolframin ER Transmembrane Glycoprotei
Wnt3a	Wnt Family Member 3A
XBP1	X-Box Binding Protein 1
ZO-1	Zonula Occludens 1

CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

*This chapter has been adapted from **Vanhove W**, Nys K, Vermeire S. Therapeutic innovations in inflammatory bowel diseases. Clin Pharmacol Ther. 2016 Jan;99(1):49-58.*

1. The paradigm of patient management in IBD

Inflammatory bowel disease (IBD) is characterized by a chronic transmural (in case of Crohn's disease) or mucosal (in case of ulcerative colitis) inflammatory infiltrate as a result of a defective immune response to enteric microbiota. Crohn's disease and ulcerative colitis are considered the two opposite phenotypes of the spectrum and both diseases have their highest incidence rates between 15 and 25 years of age. The most noteworthy advance in the treatment management of IBD of the past decades has certainly been the introduction of monoclonal antibodies directed to tumor necrosis factor-alpha (TNF-α). The role of TNF-α in IBD was indicated by several studies showing upregulation of this cytokine in stool, serum, and the intestinal mucosa of patients with IBD[1-4]. Pivotal randomized controlled trials have demonstrated efficacy in induction and maintenance of remission, in endoscopic healing, and in the reduction of surgical resections and hospitalization rates[5-11]. In August 1998 (and one year later, also in Europe), Remicade® (infliximab (IFX)), a chimeric monoclonal anti-TNF antibody, was approved by the US Food and Drug Administration (FDA) for short-term treatment of moderate-to-severe Crohn's disease in patients who did not respond to conventional therapy or had a fistulizing phenotype and, as such, IFX became the first biological agent that was approved for IBD treatment. Over the years, humanized and human antibodies were introduced, which presented improvements in the way of administration from i.v. to s.c. and in reducing immunogenicity and allergic reactions. Currently, IFX, adalimumab, certolizumab pegol, and golimumab are all approved for treatment of IBD. A recent meta-analysis by Hazlewood *et al.* compared all anti-TNFs and concluded that adalimumab and the combination of IFX with azathioprine were the most effective therapies to induce and maintain remission in patients with Crohn's disease[12]. A TNF-efficacy meta-analysis has also been performed for ulcerative colitis; overall, the results point to a slightly increased benefit of IFX over adalimumab and golimumab for the induction and maintenance of clinical response, remission, and mucosal healing[13]. Although anti-TNF agents are efficacious in a significant proportion of patients, there are several concerns. In terms of efficacy, only 30–50% of patients will achieve clinical and mucosal remission, considered to be

the goal of therapy currently, therefore leaving large margins for improvement. The long-term corticosteroid-free remission rates are even lower and do not succeed 20–30%. Second, these agents have a quick onset of action but are hindered by significant loss of response, which is largely explained by antibody formation and increased clearance rates. Third, although the overall safety profile is considered safe, there are concerns with respect to particular rare malignancies (hepatosplenic T-cell lymphoma, melanomas). However, more common are the psoriasiform skin lesions that occur in 20% of patients and may be incapacitating because of their location in the face[14].

From a mechanistic conceptual point of view, anti-TNF agents reduce the inflammatory infiltrate but do not block the underlying pathogenic triggers. Hence, treatment cessation results in recurrence of inflammation within one year in more than 50% of patients. The PREVENT study investigated if anti-TNF, when started immediately postoperative in the case of a curative resection of Crohn's disease, could prevent disease recurrence. Although the proportion of patients with endoscopic disease recurrence was significantly lower in the anti-TNF treated arm than in the placebo-treated arm, the results showed that anti-TNF is not able to fully stop disease progression[15].

All these points illustrate the need to continue the search for novel therapeutic agents. This development could be situated on various levels. First of all, there are the many compounds - monoclonal antibodies and/or small molecules - that target inflammatory cells. A different approach consists of impacting on very early events of disease pathogenesis, and stimulates or suppresses the disease depending on the target. The recent anti-sense therapy against Smad7 could be considered in this approach; but so are the various cell-based therapies. However, a truly innovative management would be one that is based on the underlying mechanisms of disease. In contrast to the general acceptance of a multifactorial etiology of the disease, treatment of IBD so far is very "homogenous" and consists of (non)-specific anti-inflammatory agents, including corticosteroids, immunosuppressive drugs (azathioprine and methotrexate), and/or anti-TNF monoclonal antibodies. It is surprising that the advances in translational knowledge about the factors triggering disease onset have not been translated to approach the disease from a molecular angle. Nevertheless, a number of key cellular pathways have emerged, including bacterial recognition, autophagy, endoplasmic reticulum

stress, and intestinal barrier function. If the therapeutic approach could be based on the underlying functional mechanisms driving the disease in a given patient, this would represent a breakthrough in the current paradigm of treating complex multifactorial inflammatory conditions and could revolutionize the paradigm of disease management.

2. Toward innovative management of IBD

The relatively high treatment failure rate of anti-TNF therapy and the emergence of new therapeutic classes highlight the need for patient selection, a more thoughtful use of anti-TNF agents, and targeting new pathways involved in IBD. In line with TNF blockade, an evident choice for IBD therapeutics is compounds that target other inflammatory mechanisms or cells. In that perspective, new cytokine antibodies or strategies inhibiting downstream cytokine signaling or adhesion molecules have shown potential and/or have been approved recently.

2.1. Anti-IL-12/23

Interleukin (IL)-12 and IL-23 are important proinflammatory cytokines belonging to the IL-12 superfamily. They are composed of the same p40 subunit allowing simultaneous inhibition of these cytokines. Their association with IBD is well accepted as both cytokines are upregulated in intestinal mucosa of patients with IBD and genetic polymorphisms in the genes encoding the IL-23 receptor as well as the p40 subunit are associated with IBD[16]. These findings are supported by the fact that IL-12 and IL-23 mitigate the differentiation of naive T cells into Th1 and Th17 cells, respectively, and thus contribute to the lymphocytic signature that is associated with IBD[17]. The phase 2 randomized, placebo-controlled trial in patients with Crohn's disease demonstrated that ustekinumab, a human monoclonal anti-p40 anti-body, was most efficacious in anti-TNF exposed patients with Crohn's disease[18]. As such, subsequent studies with this compound focused on this specific patient group and confirmed previous results[19, 20]. The efficacy of ustekinumab in anti-TNF naive patients was addressed in the large phase 3 UNITI programs: UNITI-1 was designed to study the short-term efficacy of ustekinumab in patients with Crohn's disease with failure or intolerance to anti-TNF, whereas only anti-TNF naive CD patients were included in UNITI-2. These studies gave a final confirmation that ustekinumab is equally effective regardless of previous anti-TNF exposure. Apparent differences between UNITI-1 and -2 can be explained by differences in disease

duration and the fact that disease was less refractory in the UNITI-2 population[21]. This drug has in the meanwhile been approved by the FDA and EMA to treat moderate-to-severe CD.

In contrast to ustekinumab, which allows simultaneous suppression of two cytokines, data from a phase II trial with MEDI2070 have recently been released. This human anti-IL-23 antibody that does not target IL-12 and significantly affects clinical and biological readouts in patients with Crohn's disease refractory to TNF antagonists[22]. Similar phase II results were obtained with another IL-23-selective antibody, risankizumab[23]. However, whether these compounds will outperform their competitors remains to be demonstrated.

Besides TNF-a, IL-12, and IL-23, several other cytokines have been shown to be involved in IBD and may therefore act as potential drug targets. One of these is IL-17; unfortunately, the development of the human anti-IL-17A antibody secukinumab was stopped because of absence of a clear treatment benefit and worsening of Crohn's disease in some patients[24].

2.2. Janus kinases inhibition

Inflammatory cytokine function can also be blocked by inhibiting the intracellular cytokine-mediated signals, typically via the Jak-Stat pathway. Janus kinases (Jaks) & signal transducers, and activators of transcription (Stats) are intracellular signaling molecules that mediate the signal of initial ligand-receptor binding to modulation of gene expression. The mammalian Jak family consists of four members that each transduce the signal of a specific set of cytokines and thereby are highly involved in inflammatory responses (**Figure 1**). For an overview on the Jak-Stat pathway, we refer the readers to Murray et al[25]. The relationship between Jak-Stat signaling (including their upstream ligand-receptor systems) and IBD has been extensively highlighted by animal studies, genetic association, and increased levels of cytokines that converge to Jak-Stat[16, 26].

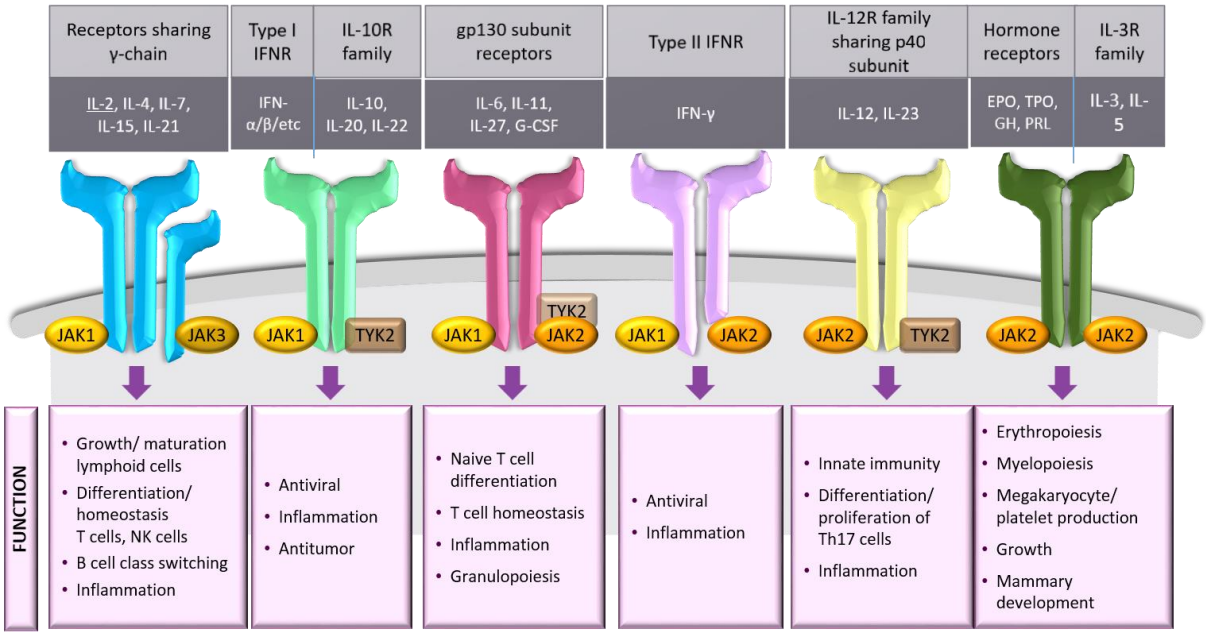

Figure 1: The JAK family plays a central role in signaling transduction for multiple growth factors as well as cytokines, including the ones that have been implicated in the pathogenesis of autoimmune diseases as based on genome wide association studies as well as mouse models of inflammation.

As Jak-Stat signaling is involved in a number of (auto)inflammatory diseases, several oral small molecules have been developed. Some of them (tofacitinib and filgotinib) have shown efficacy in preclinical studies and are currently clinically tested for the treatment of IBD[27, 28]. Tofacitinib is an oral pan-Jak inhibitor that preferentially binds to Jak1 and 3 (and Jak2 at higher concentrations), hereby diminishing the downstream effects of several IBD-associated cytokines[27]. The two phase III induction trials (OCTAVE 1 and 2) in UC showed a significant benefit over placebo with an increased infection risk as major adverse event[29]. In patients with moderate-to-severe ulcerative colitis, the oral administration of 10 and 15 mg doses resulted in significant dose-related reduction of most clinical and endoscopic disease parameters as well as inflammatory biomarker levels at week eight[30]. Because of these promising results, tofacitinib has been filed to the EMA for the treatment of UC. On the other hand, the phase II tofacitinib trial in Crohn's disease showed less convincing results, in part, because of high placebo response rates[31].

Filgotinib (GLPG0634) is a JAK1-selective (also JAK2 at higher dosage) small molecule inhibitor, potentially resulting in a better safety profile. Encouraging results of phase II trials for the treatment of Crohn's disease but also rheumatoid arthritis have been published and a large phase III program is ongoing[32, 33]. Preclinical studies showed convincing efficacy in models of

dextran sulfate sodium[28] and adoptive T-cell transfer murine colitis (presented in this thesis manuscript) with a significant improvement of symptom and histological scores.

Jak inhibitors therefore seem to be promising IBD drug candidates. Nevertheless, their safety profile when used for maintenance will be crucial. Tofacitinib could lead to unwanted blockade of erythropoietin signaling through JAK2 inhibition, aggravating anemia, which is already an IBD-related comorbidity[34]. Furthermore, as IL-10 partially exerts its immunoregulatory effect through JAK1, both agents could theoretically aggravate intestinal inflammation in a subset of patients[25, 26]. Accurate patient selection and careful dose optimization when using small-molecule Jak inhibitors in IBD is therefore recommended.

2.3. Leukocyte trafficking

Another promising idea is to dampen the influx of immune cells toward the inflamed sites in the intestinal mucosa, rather than targeting inflammatory cells and mediators that are already present. There are several arguments favoring the development of anti-leukocyte migration strategies in IBD. First of all, IBD is characterized by a strong adhesion molecule-mediated mucosal infiltration of leukocytes. Next, VCAM-1, the ligand for integrin $\alpha_4\beta_1$, is upregulated in the vascular endothelium of the inflamed gut[35]. Furthermore, MADCAM, the ligand for integrin $\alpha_4\beta_7$, which is responsible for the trafficking of lymphocytes to the gut, is also upregulated in IBD mucosa[36]. Preclinical studies showed efficacy of blocking integrin $\alpha_4\beta_7$ in the spontaneous cotton top tamarin colitis model[37, 38]. More recently, the genetic association between IBD and integrins (integrin alpha-L), chemokines, and chemokine receptors has been uncovered[16].

Anti-integrin strategies have been developed and two compounds, natalizumab and vedolizumab, are FDA approved for the treatment of IBD. Although clinical trials (ENCORE and ENACT) showed encouraging results for natalizumab (anti-α_4) in patients with Crohn's disease, in addition, leukocyte migration to the central nervous system was prevented, promoting progressive multifocal leukoencephalopathy, an often lethal disease caused by reactivation of John Cunningham virus in the brain[39, 40]. Natalizumab is only approved in the United States and not in Europe[41, 42]. A more favorable safety profile was obtained for vedolizumab, a humanized monoclonal antibody that selectively binds $\alpha_4\beta_7$ integrin, therefor selectively

targeting gut homing leukocytes. The large phase 3 GEMINI programs demonstrated superior efficacy in induction and maintenance of remission and, as of 2014, vedolizumab was approved by the FDA and the European Medicines Agency for treatment of Crohn's disease and ulcerative colitis. In contrast to the GEMINI I study in ulcerative colitis,[43] the GEMINI II program in Crohn's disease showed a slower onset of response, both for anti-TNF naive (GEMINI II)[44] and nonresponsive patients (GEMINI III),[45] indicating the need for a bridging therapy[46]. The recently published open-label GEMINI long-term safety trial data show that both UC and CD patients benefit from vedolizumab therapy in the long term[47].

Etrolizumab, a humanized monoclonal anti-β_7 antibody, demonstrated significant clinical improvement and favorable safety in a phase 2 study in patients with moderate-to-severe ulcerative colitis and failure to respond to conventional treatments[48]. Based on these encouraging results, a large phase 3 program is ongoing in which some studies also include an active comparator arm against anti-TNF agents (e.g., NCT02136069 and NCT02163759).

3. Targeting early events in IBD pathogenesis to restore intestinal homeostasis

Despite the fact that blocking leukocyte trafficking and downregulating local inflammatory responses have shown clinical benefits in IBD, these treatments cannot fully interfere with the chronic cycle of inflammation without continuous drug therapy. New approaches are needed that target initial events in intestinal inflammatory pathogenesis in order to reset the mucosal immune system to a state of homeostasis. A provocative study published in the New England Journal of Medicine earlier this year indicated that this may be achieved by antisense-mediated targeting of disturbed TGF-signaling. Likewise, cell-based therapies may also hold this promise.

3.1. Anti-Smad7 (TGF-β/BMP signaling) and other antisense oligonucleotides

TGF-β is a pleiotropic cytokine involved in embryonic development, but it is also responsible for cellular homeostasis by exerting anti-inflammatory effects. In short, binding of TGF-β to its type II receptor causes recruitment and activation of a type I receptor, which, in turn, phosphorylates the second messenger complex, SMAD2/3. SMAD2/3-P recruits and binds SMAD4, forming a complex which will translocate to the nucleus where it acts as a transcription factor eventually leading to downregulated nuclear factor-kappa B signaling after exposure to inflammatory cytokines, and thus tempering the inflammatory response[49].

SMAD7 is a negative regulator of TGF-β signaling by binding to the type I receptor, hereby interfering with SMAD2/3 phosphorylation[50]. Both TGF-β and SMAD7 proteins are upregulated in IBD mucosa, accompanied by a reduction in SMAD3 phospho-rylation[50, 51]. These observations have been confirmed in animal models of TNBS and oxazolone-induced colitis. Furthermore, administration of oral anti-SMAD7 oligonucleotide, interfering with SMAD7 mRNA translation, reduced colitis in mice[52]. These findings led to the development of Mongersen®, an oral anti-SMAD7 oligonucleotide, for the treatment of IBD. The phase II trial by Monteleone *et al.*, in which a two-week administration of Mongersen® led to a clinical response that was sustained for over three months, indicated a very long-term treatment effect[53]. The results of this study needed confirmation by studies that included endoscopy and thus recently the efficacy of Mongersen® was also confirmed at an endoscopic level in a small open label study by Feagan *et al.*[54, 55]. Nevertheless, there are is still a need for properly sized, placebo controlled studies that include endoscopy-based endpoints. Also, safety concerns have been raised as increased TGF-β signaling may induce fibronectin and collagen synthesis leading to fibrosis and stenosis of the bowel[54]. This was not observed during a phase I trial with six months of follow-up, as indicated by small intestine contrast ultrasonography and serum markers of fibrosis; although such side effects can only be excluded after long-term observation[56]. Nevertheless, the rate and longevity of clinical improvement in the Monteleone *et al.* study of 2015 strengthens the hypothesis that this drug can actually reset the mucosal immune system and has given many gastroenterologists high expectations[53].

Binding (and thereby degradation of) target mRNA with (orally administrable) antisense molecules allows significant and specific inhibition of the involved pathway (**Figure 2**). It is therefore not surprising that antisense molecules targeting other players in IBD pathogenesis have been developed and clinically tested.

Alicaforsen is an intercellular adhesion molecule-1 antisense oligonucleotide designed for rectal (local) administration in patients with ulcerative colitis. Intercellular adhesion molecule-1 is a transmembrane protein that is expressed on many cell types involved in intestinal inflammation such as epithelial, endothelial, and immune cells[57]. It is involved in leukocyte migration as well as cell-to-cell immune signaling. Suppressing intercellular adhesion molecule-1 should therefore act on intestinal inflammation at multiple levels. This might explain why clinical studies in patients with ulcerative colitis have shown both long-term and

short-term efficacy[57, 58]. The topical administration route of Alicaforsen has also led to a successful open-label trial in patients with pouchitis and a phase III program is ongoing (NCT02525523)[59]. However, it showed no efficacy when administered i.v. to patients with Crohn's disease[60].

The class of antisense oligonucleotides (ASOs) is generally considered as safe, however, preclinical studies indicated slight histological and functional changes in the kidneys of monkeys that were treated with higher doses of ASO, these alterations co-occurred with the accumulation of ASO molecules in the kidney[61]. These findings might explain a case of acute kidney injury with renal ASO accumulation in a 56-year-old woman who was treated with an ASO directed against PCSK9 and will need to be further investigated[62].

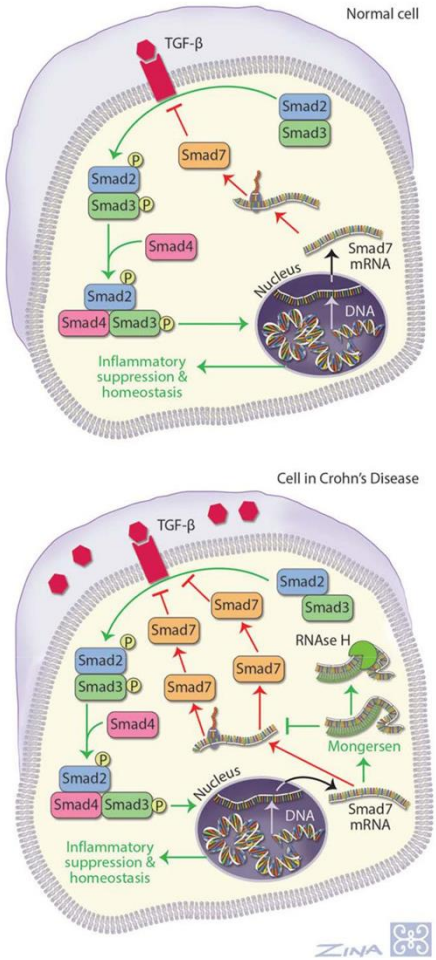

Figure 2: The TGF-b1 receptor transduces its signal by phosphorylating SMAD2 and 3 after which it will form a complex with SMAD4 and translo-cate to the nucleus where they will act as a transcription factor. The phos-phorylated SMAD complex will alter transcription, leading to inflammatory suppression, and promoting cellular homeostasis. SMAD7 is a negative regulator of TGF-b signaling by interacting with the receptor and thereby preventing SMAD2/3 phosphorylation after initial binding of the ligand. Mongersen interferes with this inhibitory process by selectively binding SMAD7 mRNA and hereby preventing translation and promoting RNAse H dependent degradation of the mRNA molecule.

3.2. Cell-based therapies

Another highly promising concept in IBD management has been the introduction of cell-based therapies (i.e., the administration of *ex vivo* expanded/manipulated (stem) cells that have immunosuppressive effects and that are derived from the patient's own tissue (autologous) or from HLA-matching donors (allogeneic))[63]. Allogeneic and autologous hematopoietic stem cell transplantation has been used successfully in oncology. When used in patients who had concomitant Crohn's disease, this treatment induced remission, which was believed to result from profound immunosuppressive and tissue-repair inducing properties[63, 64]. Results of the Autologous Stem Cell Transplantation International Crohn's Disease trial indicate that the promising treatment efficacy cannot only be explained by pretransplant conditioning. Nevertheless, the mortality risk associated with bone marrow transplantation makes this approach unfavorable as treatment for nonlethal diseases, such as IBD.

Mesenchymal stromal cells refer to several bone-marrow derived proliferative cell types that can differentiate into a wide variety of nonhematopoietic cells *in vitro*. These cells inhibit cellular inflammatory responses and induce an immunotolerant phenotype in the gut[65-67]. Their wide distribution throughout the body and their preliminary efficacy in Crohn's disease without immune suppression or bone marrow depletion makes mesenchymal stromal cells therapy an ideal alternative to bone marrow or stem cell transplantation. Phase I and II studies in moderate-to-severe Crohn's disease showed promising results and high potential to reset the intestinal mucosa into immunologic homeostasis[66-68].

Alternative or more easily available cell sources are being tested for mesenchymal stromal cells therapy. In 2009, a phase II study showed that autologous adipose-derived stem cells could successfully heal complex perianal fistulas in both patients with Crohn's disease and patients without Crohn's disease[69]. Administration of allogeneic adipose-derived stem cells also showed efficacy in phase II[70] and III[71] trials and has led to the FDA approval of Cx601 for the treatment of complex perianal fistulas in Crohn's disease. European Medicines Agency approval is expected in the first quarter of 2018. In 2012, Desreumaux *et al.*[72] published the results of the first clinical trial showing safety and efficacy of i.v. administration of *ex vivo* expanded, autologous blood-derived regulatory T lymphocytes (Ovasave) to patients with refractory Crohn's disease[72] but the phase IIb study was temporary halted (NCT02327221). An overview of cell-based therapies that have already been clinically tested is given in **Table 1**.

Table 1: An overview of cell-based therapies that have already been clinically tested in IBD management

Cell type	Source	Administration route	Allogeneic/autologous	Indication	Adverse events	Advantage
Hematopoietic stem cells	Bone marrow	Intravenous	autologous	Luminal disease	Serious infections, malignancy	Replaces immunogenic lymphocytes by immunotolerant ones
			allogeneic	Luminal disease	Serious infections, malignancy, GvHD	Lymphocytes replaced by cells without genetic susceptibility
Mesenchymal stromal cells	Bone marrow	Intravenous	Both	Luminal disease	Transient fever	No pre-transplantation immune ablation needed
	Adipose tissue	Local application	Both	Fistulizing disease	Minimal	Easily obtainable, no pre-transplantation immune ablation
	Umbelical chord	Intravenous	allogeneic	Luminal disease	Minimal	
Regulatory T cells	Antigen specific regulatory Tcells	Intravenous	autologous	Luminal disease	Immune response against Tcell Drosphila antigen	

4. Future prospects in IBD management

4.1. A mechanism-based approach

Given the multifactorial etiology of IBD and the heterogeneity in clinical phenotypes, a management system based on the key cellular pathways triggering or contributing to disease onset should be attempted. It is surprising that the advances in knowledge about the molecular factors have not been translated to approach the disease from a molecular angle. Such a molecular angle will allow us to functionally characterize the main pathways identified in IBD in a given patient and correlate this to meaningful outcomes. This seems a more intelligent approach but has never been explored. Moreover, this would represent a breakthrough for treating complex multifactorial inflammatory conditions in general.

The current hypothesis in IBD is that the disease results from a faulty immune recognition, tolerance, and defense against the commensal microbiota and dietary antigens present in Westernized diets and in a genetically susceptible host[73]. The genetic progress has been instrumental thanks to collaborative efforts led by the international IBD Genetics Consortium. Of the more than 200 IBD susceptibility loci identified today by genome-wide association studies and subsequent meta-analyses, key cellular pathways have emerged, including bacterial recognition, autophagy, endoplasmic reticulum stress, and intestinal barrier function[16, 74]. Probably the largest theme resulting from genetic studies in IBD is that a deficient bacterial recognition and handling underlies disease pathogenesis. This was first demonstrated by the identification of NOD2/CARD15 mutations in 2001[75, 76]. Subsequently, murine models of colitis and human studies linked mutations in this gene to defective Paneth cell function and defensin secretion[77, 78].

A second important pathway in disease pathogenesis relates to intestinal barrier function. The gut mucosa is constantly exposed to a huge burden of dietary antigens, diverse microorganisms, and external compounds. Its ability to act as a barrier against the passage of potentially harmful molecules is therefore critical for normal homeostasis. The single cell layer of the intestinal epithelium constitutes the primary determinant of intestinal barrier function, along with the mucus layer and immunity-related responses (innate immune cells and secreted proteins)[79]. A primary defect in barrier function in IBD is underscored by animal colitis models, in which increased intestinal permeability precedes disease expression by weeks to

months[80]. Moreover, in clinically asymptomatic patients with Crohn's disease, an impaired permeability was found to precede clinical relapse and the onset of symptoms by up to one year. These barrier defects also exist in a subset of unaffected family members of patients with Crohn's disease[81]. An interesting case described a 21-year-old girl with a strong family history of IBD who had an increased intestinal permeability at least eight years before the diagnosis of Crohn's disease[82]. In addition, human genome-wide association studies identified several variants in epithelial cell integrity genes. Endoplasmic reticulum stress-induced signaling (the unfolded protein response, UPR) and autophagy are essential for maintaining normal cellular homeostasis and appropriate immune/inflammatory responses in the human body[83-85]. The UPR is a stress response mechanism essential for cell homeostasis which is activated by an accumulation of un- or misfolded proteins in the endoplasmatic reticulum (ER), a status called ER stress. Activation of the UPR will lead to translational attenuation, induction of chaperones and increased proteasomal degradation in order to reduce the protein load and increase the protein folding capacity of the ER. Defects in the UPR machinery or prolonged ER stress can lead to inflammation and even apoptosis (**Figure 3**). As such ER stress/UPR can be directly involved in the pathogenesis of IBD by interfering with protein secretion (mucus proteins, anti-microbial factors and cytokines), by causing defects in the epithelial barrier of the gut and by responding inadequately to commensals or pathogens[84, 86, 87]

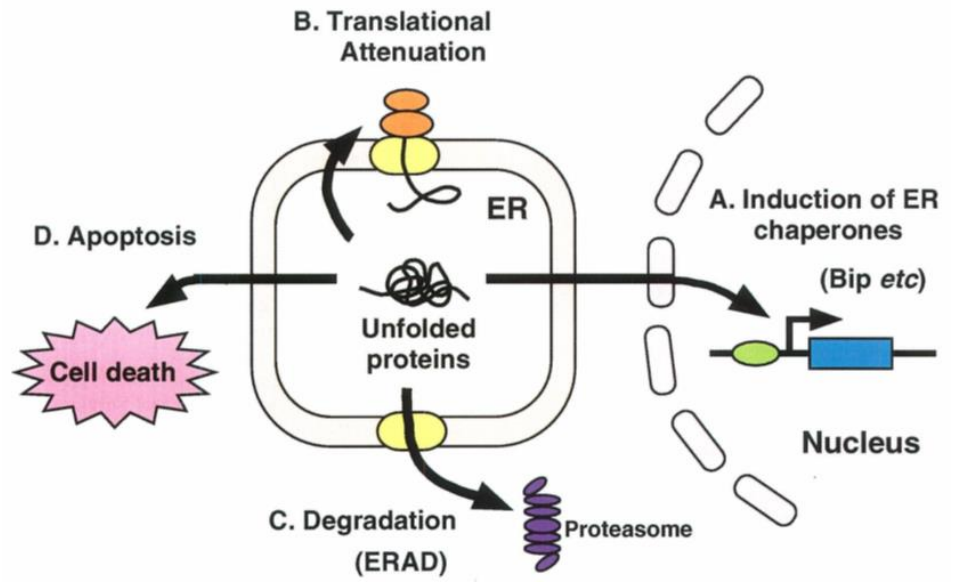

Figure 3: Accumulation of unfolded or misfolded proteins in the ER activates the unfolded protein response (UPR) which comprises four distinct cellular processes: (A) Transcriptional induction of ER chaperones increases protein folding activity and prevents protein aggregation. (B) Translational attenuation reduces the load of new protein synthesis and prevents further accumulation of unfolded proteins. (C) The ER-associated degradation (ERAD) pathway eliminates misfolded proteins by the ubiquitin-proteasome system. (D) Unresolved ER stress (eg. when the UPR is severely impaired) can eventually lead to apoptosis. Figure reproduced from Araki et al.[88]

Autophagy is an important well-conserved cellular clearance mechanism [89, 90]. This process contributes to homeostasis by provision of essential nutrients in times of nutrient deprivation. Autophagy also has several immunologic functions by aiding in the clearance of intracellular microbes or organelles, antigen presentation, inflammasome inhibition and cytokine secretion (**Figure 4**). Hence, defects in this pathway can disturb the innate as well as the adaptive immune response to microorganisms and therefore contribute to the pathogenesis of IBD[85, 89, 91-94].

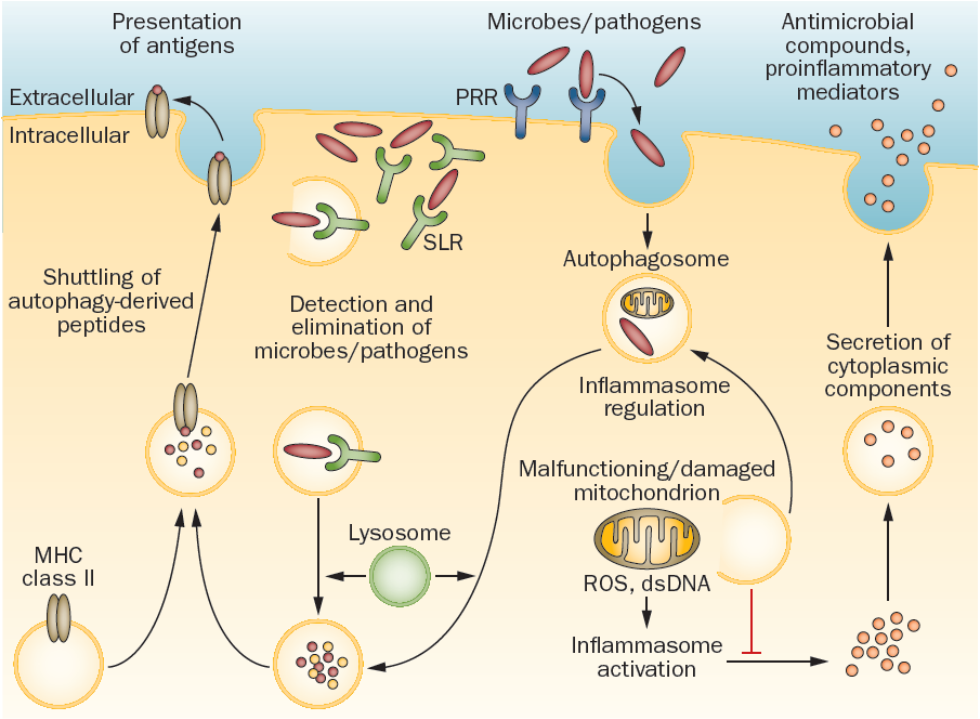

Figure 4: Autophagy interacts with essential steps in the immune response. This catabolic process aids in the direct detection and elimination of microbes and/or pathogens. Upon activation of PRRs autophagy might be induced as an effector mechanism, while intracellular SLRs directly target microbes and/or pathogens for autophagosome encapsulation. Malfunctioning or damaged mitochondria release ROS and mitochondrial dsDNA, which can activate inflammasomes resulting in the production of proinflammatory mediators. Autophagosomal removal of these malfunctioning organelles thereby regulates or inhibits inflammasome activation. Autophagy might also aid in the unconventional secretion of cytoplasmic components such as antimicrobial compounds and/or proinflammatory mediators. Finally, autophagosomes fuse with lysosomes to degrade their content (microbes, pathogens and endogenous cytoplasmic content). The generated peptides can subsequently be shuttled towards the cell membrane for MHC class II-based antigen presentation resulting in regulation of adaptive immune responses. Abbreviations: dsDNA, double-stranded DNA; PRR, pattern recognition receptor; ROS, reactive oxygen species; SLR, sequestosome 1/p62-like receptor. Figure reproduced from Nys et al.[89]

Both the ER stress and the autophagy pathways can interact with inflammasomes. These are important immune regulators for intestinal homeostasis that allow recruitment and activation of the inflammatory caspase-1 upon stimulation of one of the inflammasome-sensors by a wide variety of intracellular danger and pathogen associated molecular patterns (DAMPs &

PAMP respectively). Caspase-1 activation will lead to the maturation and secretion of IL-1β and IL-18, which are important inflammatory mediators. Furthermore, activated caspase-1 can also trigger pyroptosis, a specific form of cell death associated with inflammation. In contrast to apoptosis, pyroptosis will release cytoplasmic components into the extracellular space which can fuel inflammatory processes in adjacent cells. Inflammasome activation can have homeostatic functions in the intestinal epithelium by providing adequate amounts of IL-18 which is needed for intestinal cell proliferation and barrier function. In contrast, inflammasome activation in the lamina propria (LP) has detrimental effects as both IL-18 and IL-1β will induce production of other pro-inflammatory cytokines and chemokines leading to intestinal inflammation[95-100] (**Figure 5**).

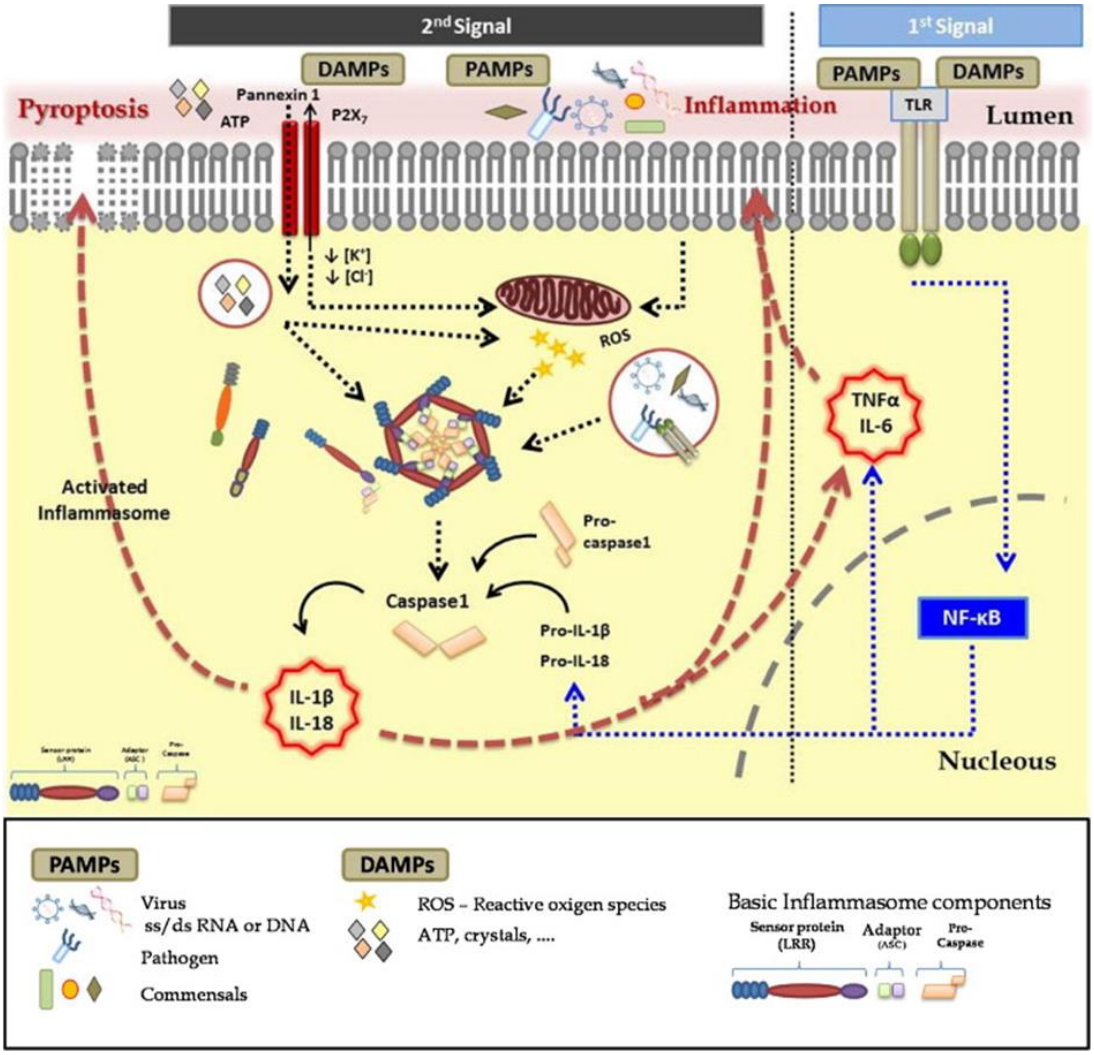

Figure 5: Components of the inflammasomes, their assembly and activation. Representative image showing the general mechanisms of activation of the inflammasomes largely based on NLRP3 inflammasome signaling. A large variety of endogenous and exogenous (microbial) molecules (DAMPs and PAMPs) can induce the inflammasome cascade. Two signals are needed to secrete the inflammasome related cytokines IL-1β and IL-18 and other cytokines (such us IL-6 and TNFα), one comes from TLRs and the other one from the cytoplasmatic NLRs to, at the end, induce inflammation or pyroptosis. Figure reproduced from Aguilera et al.[97]

These pathways are perturbed in IBD by demonstration of genetic defects in *XBP1* and *ORMDL3* (ER stress), *IRGM*, *ATG16L1*, and *LRRK2* (autophagy) and *NLRP3* (inflammasome)[16, 101]. Additionally, our group also added *ULK-1* (*ATG1*) to the list of autophagy-related Crohn's disease susceptibility genes[102, 103]. Interestingly, ER stress, autophagy and inflammasomes seem to be cross-linked. Several studies have shown that the UPR can activate autophagy mechanisms, implicating that defects in the autophagy machinery can lead to an increased ER stress and vice versa[93, 103, 104]. Moreover autophagy has an inhibitory effect on initial inflammasome activation, however it has a promoting effect on the secretion of inflammasome effectors IL-1β & IL-18[99].

A concerted unfolded protein response and/or autophagy activation might represent an innate mechanism to sense and appropriately respond to threatening changes of the mucosal microbial environment. As a consequence, (genetic) events causing imbalances in endoplasmic reticulum stress maintenance and/or normal autophagy are important drivers of human inflammatory diseases, like IBD. Preclinical studies have been performed with the conjugated bile-acid tauroursodeoxycholic acid and to a lesser extent 4-phenylbutyrate, which are both chemical chaperones and thus are able to resolve endoplasmic reticulum stress. These were efficacious in reducing acute and chronic dextran sulfate sodium-induced colitis, but also spontaneous colitis in IL-10 knockout mice[87]. Furthermore, Laukens *et al.* showed that clinical signs of dextran sulfate sodium-induced colitis are preceded by events that trigger epithelial apoptosis, such as caspase-3 upregulation and Bcl2 downregulation[105]. These events were prevented or reduced in tauroursodeoxycholic acid treated mice. As for autophagy, several of the already established IBD treatments (including anti-TNF therapy) already influence autophagic processes[89]. In 2008, the clear association of defective autophagy and IBD had led to the first successful clinical use of the chemical autophagic upregulator rapamycin (Sirolimus®) in a patient with failure to respond to standard treatments.[106] More recently, Mutalib *et al.* showed in a small study that the same drug was effective to treat children with severe refractory IBD[107]. These still limited results support the initiation of larger clinical trials and the development of drugs that upregulate autophagy and/or reduce endoplasmic reticulum stress in the intestinal mucosa.

Finally, the microbiota plays a pivotal role in the onset and perpetuation of IBD, as mentioned previously. Intestinal microbiota is essential for the development of inflammation in colitis

animal models as germ-free mice do not develop colitis[108]. Our group has contributed to the literature in this field by showing that the gut mucosa remains intact after diversion of the fecal stream in humans, whereas after exposure of the gut to luminal contents, recurrence of inflammation is observed[109]. The overall composition of the gut microbiota and the presence or absence of specific species is important for homeostasis and tolerance of the immune system. Metagenomics identified three human enterotypes based on the microbiota composition, each driven by different genera[110]. Patients with IBD have fewer anti-inflammatory bacteria and more proinflammatory bacteria; this imbalance is also described as dysbiosis. A qualitative and quantitative reduction of the firmicutes and bacteroidetes phyla and concomitant increase of proteobacteria has repeatedly been demonstrated in IBD[111, 112]. A reduction of *Faecalibacterium prausnitzii* is the most replicated species-specific finding so far in Crohn's disease and is confirmed both in fecal and mucosal samples. This species has anti-inflammatory and immunomodulatory effects *in vivo* and *in vitro*. In addition to *F. prausnitzii*, the adherent invasive *Escherichia coli* is increased in the ileal mucosa of patients with Crohn's disease and may sustain inflammation[113]. In the meantime, such dysbiosis is also well-described in ulcerative colitis. We recently identified that *Roseburia hominis* and *F. prausnitzii*, both butyrate-producing bacteria of the Firmicutes phylum, are less abundant in patients with ulcerative colitis[114]. A recent study in pediatric Crohn's disease could even discriminate the patient's phenotype based on mucosal dysbiosis[115]. The authors generated a dysbiosis index that presents itself as a gradient (percentage of disease-associated organisms) across the patient population. As such, controlling the microbial composition may be an essential part of IBD management. Several approaches have been developed that alter the microbial composition of the gut toward more anti-inflammatory enterotypes (e.g., via dietary advice, pre/ probiotics, and fecal microbiota transplantation), which has gained large interest in the last few years, illustrated by the large number of active clinical trials (e.g., NCT02335281, NCT02033408, and NCT02390726).

4.2. Toward personalized IBD management

Not all pathways are believed to be equally important in all patients and contributing to disease in the same extent. A qualitative (which pathway?) and/or quantitative (how much defective?) estimate of the different measurable factors triggering disease in a given patient has, to our knowledge, never been attempted. Nevertheless, we feel the expertise and

technology is there to do it, but the challenge lies in the necessity of applying an integrative approach using various patient materials (DNA, inflammatory cells, mucosal biopsies, and stool) and techniques. However, we think it is the smartest way to really revolutionize management of disorders with a multifactorial etiology like IBD (**Figure 6**).

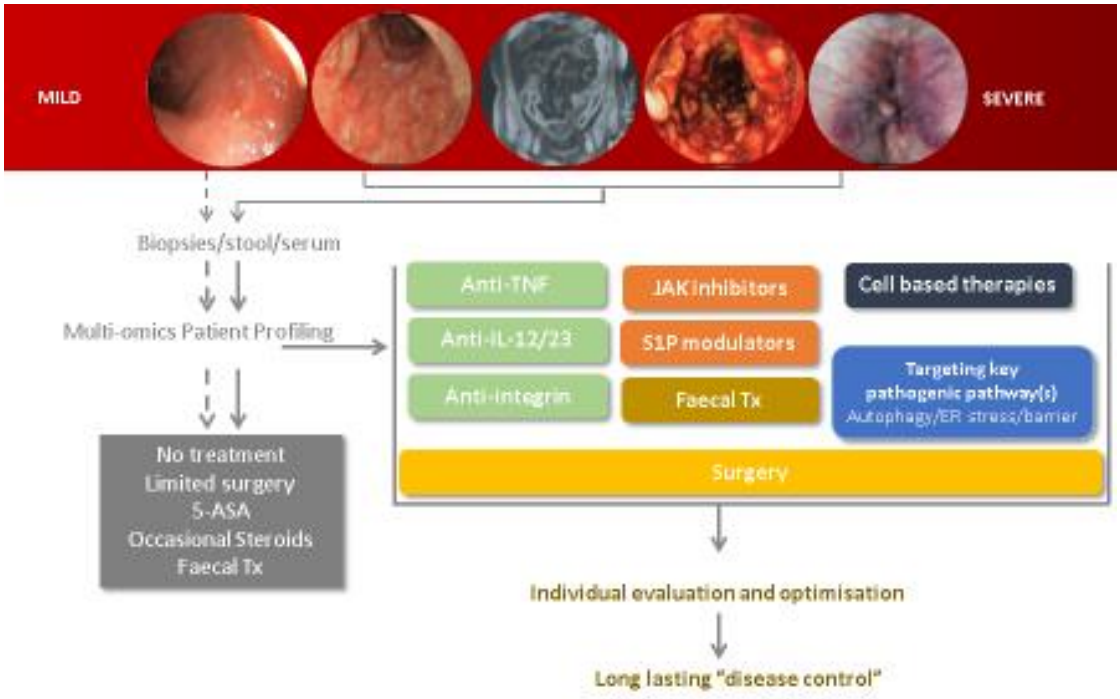

Figure 6: Changing the paradigm of IBD treatment toward a personalized and mechanism-based approach.

However, personalized medicine truly implies an individualized approach that starts at diagnosis and has the potential to aid in prognosis but should also apply to management. This last aspect has certainly not been the case in IBD, and although attempts have been made to identify biomarkers for differentiating Crohn's disease from ulcerative colitis and differentiating subgroups of patients with a different outcome, the only successful application of personalized medicine in IBD at present is the therapeutic drug monitoring of azathioprine and anti-TNF agents[116, 117].

That being said, a personalized therapeutic approach is very well known and applied already in other disease areas. In oncology, gene expression profiling has been successfully used to identify transcriptional signatures that predict several aspects of disease behavior, including risk of metastasis and response to chemotherapy[118, 119]. These gene expression-based biomarkers have also been translated into clinical practice and have received FDA approval[120]. In contrast, in autoimmune and inflammatory disorders, such techniques have generally not detected signatures with equivalent prognostic utility. Typically, the tissues examined

(peripheral blood mononuclear cells, mucosal biopsies...) are heterogeneous, and, hence, any transcriptional variation detected could predominantly reflect differences in the cellular composition between samples. Researchers from the University of Cambridge identified a transcriptional signature in separated CD8+ T cells, which predicted prognosis in patients with Crohn's disease and patients with ulcerative colitis[121]. Interestingly, the same signature was previously also found to predict disease prognosis in systemic lupus erythematosus and in patients with anti-neutrophil cytoplasmic antibody-associated vasculitis. There was a higher incidence of relapsing disease in the subgroup of patients who had an elevated expression of genes involved in antigen-dependent T cell responses, including signaling initiated by both IL-7 and TCR ligation. The fact that this signature could be found across several autoimmune and immune-mediated diseases suggests that, although being distinct autoimmune and inflammatory conditions, the course of these diseases may be influenced by common pathways. These subgroups, which can be identified by measuring expression of just three genes, raise the prospect of individualized therapy and suggest new potential therapeutic targets in autoimmunity. A large prospective randomized study is soon to start in the United Kingdom to validate these findings. Additionally, our group recently reported specific metagenomic signatures for Crohn's disease and ulcerative colitis differentiating them from healthy individuals. We also identified a mucosal gene signature to predict response to IFX in IBD colitis (patent application #EP20090724342)[122, 123].

5. Conclusion

The imminent introduction of new therapeutics marks promising times in IBD management. Recent approvals and novel therapies hopefully awaiting approval offer alternatives to the existing armamentarium. Some of these treatment options target early molecular events in disease pathogenesis and promise a long-lasting disease-suppressing effect. However, additional efforts are needed to improve patient categorization and select the best treatment strategy. Ten to 20% of patients cannot be classified to Crohn's disease or ulcerative colitis, second, there is considerable inter- and intra-patient variability in the disease course, and good prediction models are lacking[124]. Also, from a therapeutic point of view, there is considerable room for improvement. One of the reasons for this may be that the registered therapeutic options (compared to rheumatologic diseases, for instance) are limited. However, more importantly, we feel that an essential explanation lies in the fact that the approved drugs

exert their effect by suppressing the end result of chronic inflammation in a nonselective way. A molecular approach of patients with IBD from diagnosis over prognosis to therapy could revolutionize the paradigm of disease management for IBD, but, potentially, also many other chronic inflammatory disorders characterized by a multifactorial pathophysiology.

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CHAPTER 2

RESEARCH OBJECTIVES

CHAPTER 2: RESEARCH OBJECTIVES

The major issue with IBD is the huge inter-patient variability and the large proportion of patients that do not respond to therapy or develop side-effects. Therefore, there is still an unmet need for novel therapies that target other inflammatory pathways. Over the last decade, genetic association studies have uncovered cellular mechanisms such as bacterial recognition, autophagy, ER stress, JAK/STAT-signaling, etc. that have been shown to play a role in IBD pathogenesis. These findings have spurred the development of novel therapeutic compounds of which some have recently been approved (vedolizumab and ustekinumab) and more are to come (Mongersen® and tofacitinib). These novel therapies are of help in expanding treatment options for patients, but also imply that clinicians need to choose the right therapy from an expanding armamentarium. In this time of blooming therapeutic diversity, we should evolve towards a personalized approach that is based on the relative contribution of disease-driving pathways in a given patient. We believe that this pathway-based patient stratification approach will be useful for therapeutic decision making but also disease course/phenotype prediction.

The **general aim** of this PhD thesis is to improve outcome for patients with IBD by investigating in a preclinical model how the class of JAK inhibitors work and by exploring how the underlying pathophysiologic pathways could be better characterized in patients with IBD.

- The objective of the **first part** of this PhD thesis was to optimize the T cell transfer model of chronic colitis at our lab and to use this model to investigate the efficacy and mode of action of a new JAK1 inhibitor, filgotinib/GLPG0634 (**CHAPTER 3**). This project was part of a larger project in collaboration with Galapagos N.V. and Ghent University. We anticipate that the combination of preclinical and clinical data (ongoing large phase 3 program) will provide sufficient evidence for the regulatory authorities for the eventual approval of filgotinib for the treatment of IBD.

In the **second part** of this PhD we aimed to explore new ways to characterize underlying pathophysiologic pathways in IBD patients.

- We first aimed to develop and characterize a novel method to culture biopsy-derived intestinal epithelial cell (IEC) monolayers to then characterize patient-specific

epithelial defects at a functional level. As a proof of concept, we attempted to use this model to translate the genetic risk in ER stress, autophagy and inflammasomes into functional readouts (**CHAPTER 4**). We expect that the genetic risk in a given pathway carried by patients will at least influence some of the functional readouts in these pathways. These results could then guide towards a more personalized therapeutic approach to be tested in a randomized controlled setting.

- Our final aim was to investigate the effect of disease activity and infliximab treatment on the expression of ER stress, autophagy and inflammasome genes in the colonic mucosa of IBD patients (**CHAPTER 5-6)**. Given the well-evidenced involvement of these three pathways in IBD and TNF-α signaling, we expect their gene expression profiles will vary in different patient groups. Furthermore, by investigating multiple genes within a pathway we might be able to discover which key genes/proteins are dysregulated in IBD and potentially discover underlying mechanisms that lead to non-response to infliximab. These findings could have value by providing novel therapeutic targets, as transcriptomic biomarkers for disease activity and disease course or for therapeutic decision making.

PART I

IN VIVO TARGETING OF THE JAK-STAT PATHWAY IN IBD

CHAPTER 3

SELECTIVE INHIBITION OF JANUS KINASE 1 (JAK1) WITH FILGOTINIB REVERSES PATHOGENIC PROCESSES IN PRECLINICAL MODELS FOR IBD

CHAPTER 3: SELECTIVE INHIBITION OF JANUS KINASE 1 (JAK1) WITH FILGOTINIB REVERSES PATHOGENIC PROCESSES IN PRECLINICAL MODELS FOR IBD

This chapter describes the optimization of an experimental colitis model after which it was used to investigate the efficacy of filgotinib. This study was part of a larger multi-center project of which the manuscript is being prepared for submission in Gastroenterology: Nys K, Merciris D, Laukens D, Vayssière B, ***Vanhove W****, Ongenaert M, Auberval M, De Vriendt V , Monjardet A, Borgonovi M, Lepescheux L, Dupont S, Clément-Lacroix P, De Vos S, De Vos M, Brys R, Vermeire S, Galien R. Selective inhibition of Janus kinase 1 with filgotinib reverses pathogenic processes in preclinical models for IBD*

1. Introduction

Inflammatory bowel diseases (IBD) are chronic inflammatory relapsing and remitting disorders of the gastrointestinal tract manifesting as Crohn's disease (CD) or ulcerative colitis (UC). IBD are disabling conditions whose main symptoms are diarrhea, abdominal pain and rectal bleeding[1]. The fact that 20% of UC and over 60% of CD patients require surgical intervention within 10 years after diagnosis underscores the remaining unmet therapeutic need for IBD, despite the extended pharmacological armamentarium currently available[2].

The pathogenesis of IBD is considered multifactorial, with a combination of events ultimately leading to an abnormal immune response against microorganisms of the intestinal flora in genetically susceptible individuals[3]. This immune response is the target of many drugs that have been assayed for the treatment of IBD[4]. One of the prime advances of the past decades was the neutralization of tumor necrosis factor alpha (TNFα). However, even TNFα blockade ultimately establishes a stable remission in less than 50% of patients[5].

Due to their essential roles as signal transducers downstream of cytokine receptors, the Janus Kinase (JAK) family of tyrosine kinases have been proposed as valuable targets for treatment especially in UC, and phase 2 trials with tofacitinib have been completed successfully[6]. The JAKs are non-receptor tyrosine kinases constituting a family of 4 proteins, named JAK1, JAK2, JAK3 and TYK2 that interact as pairs with many receptors for cytokines and hormones[7]. The JAKs are able to activate signal-transducer-and-activator-of-transcription (STAT) proteins that once phosphorylated dimerize and translocate to the nucleus to activate the transcription of cognate genes[8]. The receptor / JAK combinations allow a diversity of signaling modules that is further increased by the existence of 6 STAT factors that function as dimers, whose composition directly depends on the JAK pairs involved. The diversity resulting from these combinatorial options allows a high specificity in ligand effects with a limited number of proteins involved.

Several JAK inhibitors (JAKINIBs) are under development. Less selective compounds like tofacitinib (JAK3/JAK1/JAK2 inhibitor) or baricitinib (JAK1/JAK2 inhibitor) are marketed or in development for RA[9, 10] and UC[6].

Filgotinib (known as GLPG0634, GS-6034) is the first JAKINIB displaying a strong selectivity for JAK1 over the other JAK members[11]. Phase 2 proof-of-concept clinical trials were completed for RA, showing good efficacy and safety[12-14]. The role of JAK1 in the regulation of the polarization of TH1, TH2 and TH17 cells combined with the selectivity and safety characteristics of filgotinib prompted us to develop this molecule as a treatment for CD patients where it achieved recently a successful proof-of-concept study[15].

Filgotinib had already shown to effectively reduce colitis in the chronic DSS colitis model[16] but pre-clinical data in a T cell-driven IBD model were lacking. The T cell transfer model of colitis is such a chronic T cell-driven IBD model that is characterized by colitis with epithelial damage, transmural immune cell infiltration, diarrhea and weight loss. These symptoms are induced by intraperitoneal injection of $CD4CD45RB^{high}$ T cells from WT donor mice into syngeneic immunodeficient acceptor mice. We therefore aimed to set up a chronic murine IBD model at our facilities in order to evaluate the efficacy of filgotinib and to confirm previous findings that were obtained in the chronic DSS model.

2. Materials and Methods

2.1. Reagents

GLPG0634 (filgotinib) was dissolved in 0.5% methylcellulose and treatment was given once a day by oral gavage at a dose of 30 mg/kg. During the optimization phase of this study we included two active comparator groups that were treated once a day with 30 mg/kg tofacitinib by oral gavage or 10 mg/kg infliximab by intraperitoneal (i.p.) injection.

2.2. Mice

Female 5-7 weeks old BALB/cOlaHsd donor mice and BALB/cJHanHsd-Prkdc SCID acceptor mice were bred under specific pathogen free (SPF) conditions (Huntington Life Sciences, East Millstone, NJ, USA) and were housed at a dedicated in-house SPF animal facility according to the Federation for Laboratory Animal Science Associations (FELASA) guidelines. The animal studies were ethically approved by the Ethical Committee of the University of Leuven, Belgium.

2.3. Induction of chronic colitis and Experimental setup

Naïve $CD4^+CD45RB^{hi}$ T cells were isolated from spleens of normal Balb/c mice and adoptively transferred to immune-deficient SCID Balb/c mice as previously described[17]. In short, spleens from Balb/c donor mice were dissected aseptically dissociated and filtered (70 µm pore size). The resulting cell suspension was enriched using an EasySep Mouse naïve $CD4^+$ T cell enrichment kit (Stem Cell, Vancouver, BC, Canada). Cell sorting (>99%) for $CD4^+CD25^-CD45RB^{hi}(CD62L^+)$ and $CD4^+CD25^-CD45RB^{lo}(CD62L^{lo})$ was done on a FACS Aria II (BD biosciences, San Diego, CA, USA). Finally 400,000 TH cells/mouse were transferred to the SCID acceptor mice by i.p. injection.

2.4. Monitoring of disease

Mice were weighed twice/week and at the end of each experiment they were euthanized with sodium pentobarbital (Nembutal, Ovation Pharmaceuticals Inc. Deerfield, US) followed by cervical dislocation. During necropsy, colitis severity was quantified with four complementary scoring systems. The disease activity index (DAI, **Table 1**) was determined based on body weight loss, stool consistency and presence of blood in the stool. This scoring method has previously been described by Breynaert *et al.*, 2013[18].

Table 1: Criteria for calculating the disease activity index (DAI)

Stool	0	Normal and correctly formed
	2	Very soft but formed
	4	Unformed and completely liquid
Blood	0	No blood
	1	Blood present
Weight loss	0	Normal or weight gain
	1	0-5% weight loss
	2	5-10% weight loss
	3	10-15% weight loss
	4	15-20% weight loss
	5	>20% weight loss

The macroscopic damage score is based on the adhesion of the colon to the surrounding tissue, hyperemia and the length of moderately and severely inflamed colon (**Table 2**).

Table 2: Criteria for macroscopic damage score calculation

Macroscopic damage score		
Adhesion	0	No force is needed
	1	Significant amount force is needed
	2	Scissors are needed
Hyperaemia	0	No blood
	1	Blood present
Inflamed colon length	x1	Moderatley inflamed
	x2	Heavily inflamed

The colon weight/length ratio was also calculated and added as an objective indicator of colitis severity. Finally, histological scoring was performed on formalin-fixed, paraffin-embedded colon sections stained with hematoxylin and eosin. Colon lesion was scored as described by Perrier *et al.*[19] (**Table 3**). All analyses were performed in a blinded manner.

Table 3: Criteria for histological scoring

Mucosal architectural changes	0	None
	1	Focal and mild
	2	(multifocal or diffuse) and (mild or moderate)
	3	(multifocal or diffuse) and severe
Immune cell infiltration	0	Within normal limits
	1	Slightly increased infiltrate in LP
	2	Dense infiltrate in LP
	3	Cell aggregates in and beyond mucosa
Epithelial defects	0	None
	1	Unequivocal focal erosion
	2	Multifocal erosion
	3	Ulceration
Goblet cell loss	0	None
	1	Focal
	2	Multifocal
	3	Generalized

2.5. Statistical analysis

Statistical analysis and calculations were performed using GraphPad Prism 5.3 (GraphPad, La Jolla, CA, USA). Data are represented as medians with IQR and the p-values were obtained using a one-way analysis of variance (ANOVA) test.

3. Results

3.1. Pilot experiments

We performed a pilot experiment to see whether we were able to induce colitis at our facility and whether the reference compounds infliximab and tofacitinib, had beneficial effects in this setting. **Figure 1** illustrates how the weight of all four groups evolved the same way until week 5.5, after which the weight-curve of tofacitinib group started to diverge from the negative control group, followed by the other treatment groups in week 6.5. At the end of the experiment, only the infliximab treated mice showed reduced weight loss compared to the positive control group.

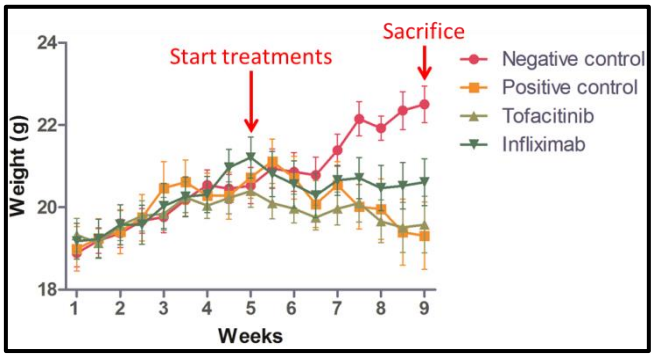

Figure 1: Pilot experiment: Weight gain/loss over time in CD4CD45RB^{lo} injected negative control mice (n=10), CD4CD45RB^{hi} injected untreated positive control mice (n=10), CD4CD45RB^{hi} injected tofacitinib treated mice (n=10) and CD4CD45RB^{hi} injected infliximab treated mice (n=9), (mean±SEM). Red arrows indicate the time of treatment and sacrifice.

The effects of T cell transfer and pharmacological treatments on inflammatory scores are shown in **figure 2**.

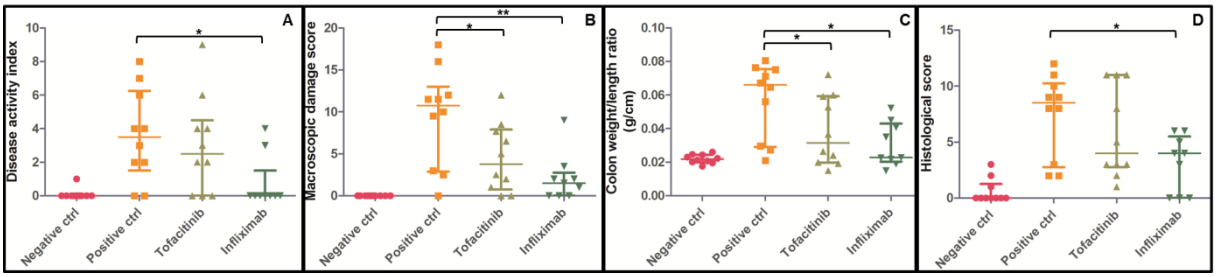

*Figure 2: Pilot experiment: Quantification of colitis severity with the disease activity index (A), macroscopic damage score (B), colon weight/length ratio (C) and histological scoring (D) of the mice described in Figure 1, (median±IQR). *p<0.05; **p<0.01*

The results indicate that we were able to successfully induce colitis in BALB/C SCID mice at our facility. Furthermore, the beneficial effects of IFX treatment on body weight were confirmed by all four scoring systems as it significantly reduced the DAI, macroscopic damage scores, colon weight/length ratios and histological scores. Also, despite the fact that it did not

influence body weight loss, tofacitinib significantly reduced the macroscopic damage score and colon weight/length ratio.

After this successful pilot experiment, we set up an identical second experiment that included GLPG0634 as a third treatment arm. During this experiment, the weight curves of all colitis groups already started to diverge from the negative control group at the beginning of week 5. IFX and GLPG0634 performed somewhat better than the positive control group from week 6 to 8 but failed to show a clear beneficial effect on bodyweight at the end of the experiment. Furthermore, the median weight of the tofacitinib treated mice was even lower than that of the positive control group were doing even worse than the positive control group (**Figure 3**).

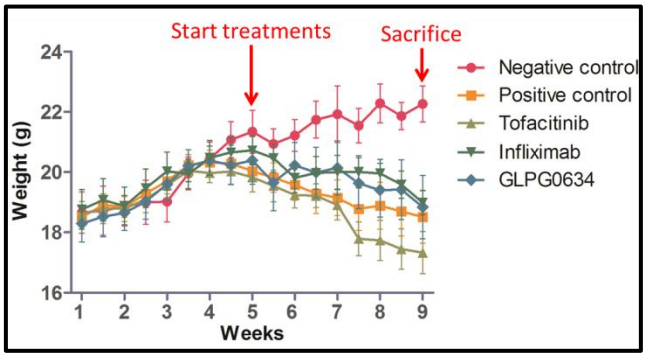

Figure 3: Weight gain/loss over time in CD4CD45RB^{lo} injected negative control mice (n=5), CD4CD45RB^{hi} injected untreated positive control mice (n=10), CD4CD45RB^{hi} injected tofacitinib treated mice (n=9), CD4CD45RB^{hi} injected infliximab treated mice (n=9) and CD4CD45RB^{hi} injected GLPG0634 treated mice (n=10), (mean±SEM). Red arrows indicate the time of treatment and sacrifice.

Figure 4 illustrates how there were also no significant differences in inflammatory scores between the treatment groups and the positive control group.

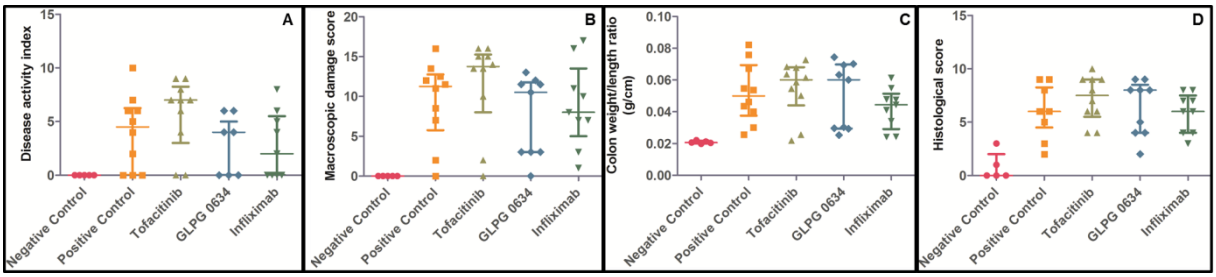

Figure 4: Experiment 1: Quantification of colitis severity with the disease activity index (A), macroscopic damage score (B), colon weight/length ratio (C) and histological scoring (D) of the mice described in Figure 3, (median±IQR).

We hypothesized that the discrepancies between this experiment and the pilot experiment were due to the earlier disease onset and thus a later time point of therapeutic intervention in the course of experimental colitis. Therefore, some of the experimental conditions were adjusted in order to counteract these problems. First of all, an additional marker (CD62L) was

used during cell sorting in order to obtain a more specific T cell subset ($CD4CD45RB^{hi}CD62L^{hi}$). We also chose to start treating the mice one week earlier and to include more mice in each treatment group. For ethical considerations, the reference compound groups (IFX and tofacitinib) were not included in this experiment.

3.2. Filgotinib reduces body weight loss and inflammatory scores

Figure 5 illustrates how $CD4^{+}CD25^{-}CD45RB^{hi}CD62L^{hi}$-injected, SCID mice started developing colitis with loss in weight starting 4 weeks after T cell transfer, in contrast to the mice injected with $CD4^{+}CD25^{-}CD45RB^{lo}CD62L^{lo}$ T cells that followed a normal growth curve. Over the course of the next 3.5 weeks the diseased mice continued to lose weight until being sacrificed at the end of week 7, while the average weight of the negative control group stabilized in time. The evolution of body weight of mice treated with GLPG0634 was intermediate between the curves of the diseased, vehicle-treated mice and the non-diseased control group. The endpoint weight of the vehicle-treated diseased group was significantly lower as compared to the negative control group. The filgotinib-treated group showed a more favorable weight evolution compared to the untreated group, displaying a significant difference at the final endpoint.

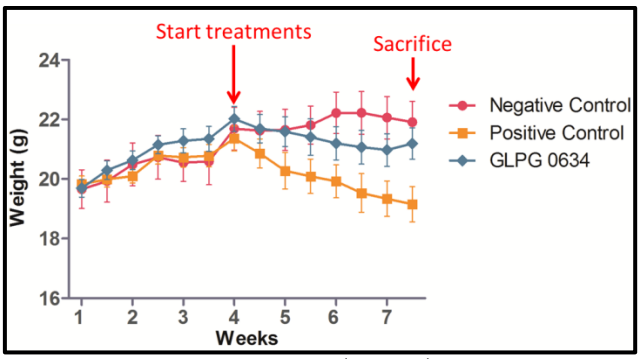

Figure 5: Weight gain/loss over time in $CD4CD45RB^{lo}CD62L^{lo}$-injected negative control mice (n=10), $CD4CD45RB^{hi}CD62L^{hi}$- injected untreated positive control mice (n=13), $CD4CD45RB^{hi}CD62L^{hi}$- injected GLPG0634 treated mice (n=12), (mean±SEM). Red arrows indicate the time of treatment and sacrifice.

In the vehicle-treated colitic mice, typical histological colonic inflammatory changes were found including goblet cell loss, infiltration of leukocytes in mucosa and submucosa, epithelial erosion, altered crypt architecture and abscesses (**Figure 6**).

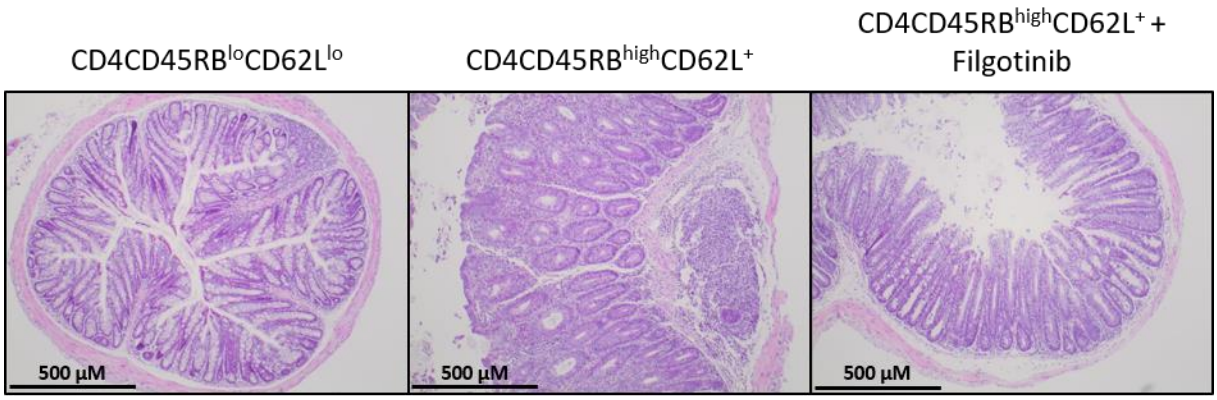

Figure 6: Representative microscopic images of the distal colon after H & E staining.

Interestingly, as compared to the diseased group, the filgotinib-treated mice showed a strong and significant improvement in the macroscopic damage score ($p<0.05$) and histological inflammation score ($p<0.01$) (**Figure 7B and 7D, resepctively**). A comparable trend, although not significant, was found when evaluating disease activity index (**Figure 7A**).

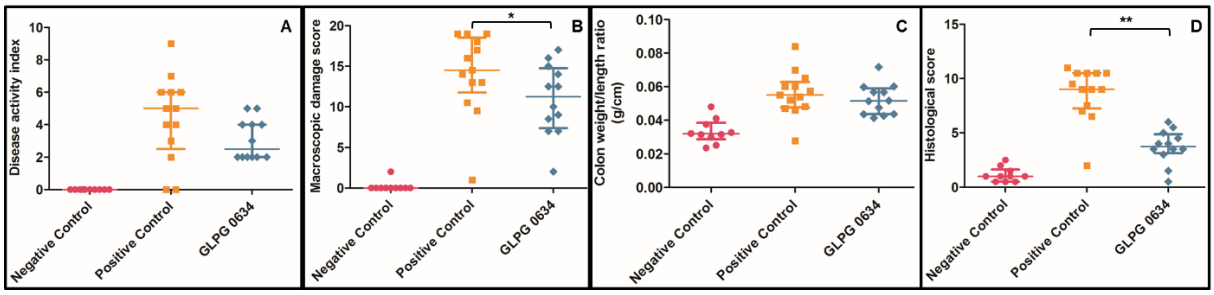

Figure 7: Quantification of colitis severity with the disease activity index (A), macroscopic damage score (B), colon weight/length ratio (C) and histological scoring (D) of the mice described in Figure 6, (median±IQR).

4. Discussion

This study was optimized and designed to answer whether the selective inhibition of JAK1 is sufficient to reduce signs of T cell-mediated colitis.

To enable to ascribe the effects of filgotinib to JAK1 inhibition, a pilot study was performed by Galapagos to ascertain that an adequate compound dose was selected for the *in vivo* studies. Analysis of the pharmacodynamic data in view of filgotinib potency in human whole blood assays (EC_{50}= 629 nM for JAK1, deduced from a IL-6-driven STAT1 phosphorylation assay and IC_{50}= 17,453 nM for JAK2, deduced from a GM-CSF-driven STAT5 phosphorylation assay) indicates that the plasma concentrations achieved in the mouse models can only support JAK1 inhibition[11].

The chronic T cell transfer colitis model is relevant to the adaptive immune system as the homing of $CD4^+$ cells to the gut ultimately leads to increased local levels of T_H1 and T_H17 cells in the absence of T_{reg} cells[20]. This model thus allowed evaluation of the efficacy of filgotinib in T cell-mediated intestinal inflammation. Filgotinib conferred structural protection to the mucosal tissue in this challenging model impacting weight loss, goblet cell loss, leukocyte infiltration in the mucosa and submucosa, epithelial erosion, altered crypt architecture and abscesses formation. Identical protection of the colon was previously found in a study by Merciris *et al.* in the chronic DSS-induced colitis model[16]. The DSS model is based on the chemical injury induced by DSS on the intestinal epithelium, leading to a strong neutrophil response. Merciris *et al.* showed that the colons from the diseased mice had a loss of normal crypt architecture which was reflected in the disease activity index (DAI) and in the decrease in colon length. Filgotinib was able to delay crypt damage and improved the DAI while maintaining colon length. Structural protection was also reflected in the gene expression analysis results, showing a reduction of the colonic expression of several key cytokines (*TNFα, IL-6, IL-12p40, IL-17A*) induced by DSS treatment. The reduction in *TNFα* and *IL-12p40* expression is of particular interest, as biologicals blocking these cytokines are part of the main therapeutic strategies for IBD treatment[5, 21]. The expression of other key markers of inflammation in IBD was reduced by filgotinib treatment, notably *Saa3* (an acute phase response protein associated with IBD disease severity)[22] and *S100a9* (a component of the disease marker in IBD patients, calprotectin)[23].

Besides inhibiting cytokines with a pathogenic character in IBD, JAK1 inhibition also inherently has the potential to inhibit the signaling of cytokines such as IL-10 or IL-22 that might be protective. However, the clear structural protection of the gut achieved by filgotinib treatment in the two mouse models, indicates that the right balance in the affected cytokines is restored by filgotinib. Importantly, the histopathological changes were confirmed at the gene expression level, where a restoration of the expression of genes associated with intestinal barrier development, such as *Pax6*, *Neurod1* or *Ptk6* was observed. In addition, other genes that are involved in IEC homeostasis and protection of the mucosal tissue against pathogen invasion, such as *Mcpt1*, *Mcpt4*, *Slpi*, and *Itln1*, were also impacted by filgotinib treatment, highlighting the protective role of the molecule in the inflamed gut[24]. Of relevance in this context, the expression of IL-33, which emerges as protective cytokine in IBD[25], was found to be restored upon filgotinib treatment. Overall, these observations are in line with the data observed in CD patients in the FITZROY study[15] and confirm that as an endpoint, filgotinib favors mucosal healing of the gut.

Histopathological data obtained after treatment with filgotinib in the two models support the fact that the compound is able to confer structural protection and impact inflammation originated by different pathogenic mechanisms. Of interest, the ability of filgotinib to inhibit the infiltration of macrophages and T cells in an inflamed tissue was also observed in arthritic joints[11]. Together, the histopathological and gene expression data obtained in two models support the JAK1 inhibition concept in the treatment of colitis and Crohn's disease.

It is of particular interest to note that the JAK1-selective inhibitors filgotinib and upadacitinib provided interesting data in patients with moderate-to-severe CD while tofacitinib, which is less selective than filgotinib and upadacitinib, failed twice in proof of concept studies in CD patients[26, 27]. These observations suggest that a certain level of selectivity may be needed to impact significantly on Crohn's disease in contrast to ulcerative colitis. Given that tofacitinib provided good results in UC[6, 28], the ongoing phase 3 study with filgotinib in UC will provide key information regarding the role of JAK1 selectivity in this pathology.

In summary, we present evidence that the JAK1-selective inhibitor filgotinib yields a strong anti-inflammatory activity in a T cell-driven mouse model of experimental colitis at efficacy exposures allowing only JAK1 inhibition. Importantly, the strong anti-inflammatory effects of

filgotinib resulted in clear structural protection. Filgotinib is currently undergoing phase 3 clinical trial development for UC and CD. Combination of biomarker analysis from these studies with data presented here is expected to allow full understanding of filgotinib mode of action in IBD.

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PART II

FUNCTIONAL TRANSLATION OF IDENTIFIED PATHWAYS IN IBD

CHAPTER 4

BIOPSY-DERIVED INTESTINAL EPITHELIAL CELL CULTURES FOR PATHWAY BASED STRATIFICATION OF PATIENTS WITH INFLAMMATORY BOWEL DISEASE

CHAPTER 4: BIOPSY-DERIVED INTESTINAL EPITHELIAL CELL CULTURES FOR PATHWAY BASED STRATIFICATION OF PATIENTS WITH INFLAMMATORY BOWEL DISEASE

This entire chapter was submitted to JCC as an original research article and has been accepted for publication: ***Vanhove W****, Nys K, Arijs I, Cleynen I, Noben M, De Schepper S, Van Assche G, Ferrante M, Vermeire S. Biopsy-derived intestinal epithelial cell cultures for pathway based stratification of patients with inflammatory bowel disease.*

1. Abstract

Background: Endoplasmic reticulum stress was shown to be pivotal in the pathogenesis of inflammatory bowel disease. Despite progress in IBD drug development, not more than one third of patients achieve steroid-free remission and mucosal healing with current therapies. Furthermore, patient stratification tools for therapy selection are lacking. We aimed to identify and quantify epithelial ER stress in a patient-specific manner in an attempt towards personalized therapy.

Methods: A biopsy-derived intestinal epithelial cell culture system was developed and characterized. ER stress was induced by thapsigargin and quantified with a BiP ELISA on cell lysates from 35 patients with known genotypes who were grouped based on the number of IBD-associated ER stress and autophagy risk alleles.

Results: The epithelial character of the cells was confirmed by E-cadherin, ZO-1 and MUC2 staining and *CK-18, CK-20 and LGR5* gene expression. Patients with 3 risk alleles had higher median epithelial BiP-induction (*vs.* untreated) levels compared to patients with 1 or 2 risk alleles (p=0.026 and 0.043, respectively). When autophagy risk alleles were included and patients were stratified in genetic risk quartiles, patients in Q2, Q3 and Q4 had significantly higher ER stress (BiP) when compared to Q1 (p=0.034, 0.040 and 0.034, respectively).

Conclusion: We developed and validated an *ex vivo* intestinal epithelial cell culture system and showed that patients with more ER stress and autophagy risk alleles have augmented epithelial ER stress responses. We thus presented a personalized approach whereby patient-specific defects can be identified which in turn could help in selecting tailored therapies.

2. Introduction

Inflammatory bowel diseases (IBD) comprise a spectrum of intestinal inflammatory conditions with Crohn's disease (CD) and ulcerative colitis (UC) as the two main entities. They are characterized by chronic inflammation of the gastrointestinal tract and are believed to result from a dysregulated immune response towards the intestinal microbiota in genetically predisposed individuals[1]. Physicians and patients still face multiple challenges as no curative treatment yet exists. A significant advance in management of IBD was the introduction of biologic agents targeting tumor necrosis factor (TNF)[2]. Almost two decades following their approval, a second and third class of biologic agents respectively targeting gut-homing $\alpha_4\beta_7$ integrin-positive T-lymphocytes and interleukins (IL)-12/23, have been added to the therapeutic options. All approved biologic agents suppress a general adaptive immune response instead of the desired targeting of underlying pathogenic triggers[3-5]. Second, steroid-free clinical remission and mucosal healing, two important treatment goals, are observed in no more than 30-35% of patients with large inter-patient variability in treatment response. The search for predictive biomarkers has been unsuccessful so far and as a consequence prediction of therapeutic success is poor[6].

Genetic association and gene/protein expression studies have uncovered novel underlying pathophysiologic pathways that are currently under (pre)clinical evaluation as a therapeutic target. IL-12/23 and Smad7/TGF-B signaling, endoplasmic reticulum (ER) stress and autophagy are accepted key players in IBD pathogenesis and are targeted by specific small molecules or antibodies that are in different stages of therapeutic development[7-18]. It is anticipated that treatment success will vary depending on which pathways drive disease in a given patient. Therefore, it will become increasingly important to identify triggers of disease in order to select the most appropriate therapy [3, 6, 19].

The intestinal epithelium is crucial for intestinal homeostasis and prevention of inflammation as this tightly connected single cell layer limits translocation of luminal microorganisms and other antigens to the lamina propria. Intestinal epithelial cells (IECs) form a physical barrier that is maintained by strong tight junction protein expression and a continuous epithelial cell proliferation in the stem cell compartment at the crypt base[20-22]. The role of the epithelial barrier in IBD is underscored by studies that associate barrier defects with disease progression

and relapse[23-25]. As described above, several epithelial cell integrity pathways such as ER stress and autophagy have been associated with IBD[21, 22, 26-28].

ER stress signaling/unfolded protein response and autophagy are two well-characterized homeostatic pathways that closely collaborate and play a key role in the innate and adaptive immune system[29-32]. Autophagy serves as an intracellular clearance mechanism for components of endogenous and exogenous origin such as mitochondria, misfolded proteins, signaling complexes and (pathogenic) microorganisms[30]. The unfolded protein response is triggered by an increased abundance of un- or misfolded proteins in the ER, also called ER stress. Prolonged or uncontrolled ER stress will eventually lead to inflammation and/or cell death[29]. Both pathways have been genetically associated with IBD; e.g. the ER stress-related *XBP1* and *ORMDL3* genes and the autophagy-related *ATG16L1*, *IRGM*, *LRRK2* and *ULK1* genes[26-28]. Nevertheless, little is known how these genetic variants functionally translate in patients with IBD. We hypothesize that inter-patient differences in the risk allele burden in these pathways will lead to distinctive functional readouts in IBD patient-derived epithelial cells.

Therefore, the aim of this study was to translate an individual's genetic risk in ER stress and autophagy into quantifiable, functional ER stress-readouts starting from patient-derived epithelial cells. To do so, we developed an *ex vivo* 2-dimensional epithelial cell culture system starting from human endoscopically-derived biopsies to quantify perturbed pathways in patients with IBD. As a proof of concept, we studied epithelial ER stress levels stratified by the number of ER stress and autophagy risk alleles.

3. Materials and methods

3.1. Patients and ethical statement

Patients with IBD followed at the IBD unit of the University Hospitals Leuven, who were genotyped as part of the international Immunochip project, were selected based on their mutations in ER stress or autophagy genes (**Table 1**, **Supplementary figure 1**)[26, 33]. We selected patients with 0, 1, 2 or 3 ER stress risk alleles and patients who had ≤3, 4, 5 or ≥6 autophagy risk alleles which was based on the risk allele distribution in the immunochipped IBD patient population at the University Hospitals Leuven (**Supplementary figure 1**). Only SNPs with a call rate > 90% and a minor allele frequency > 0.01 were included. Mucosal biopsies (8/patient) were obtained from the macroscopically non-inflamed colon of 35 patients undergoing endoscopy as part of their IBD management. Patient characteristics are provided in **table 2**.

Table 1: IBD-associated ER stress and autophagy genes with their specific SNP-IDs, chromosome n°, risk alleles and SNP locations.

Pathway	Gene	Studied SNP	Chromosome	Risk allele	Location
Autophagy	*ATG16L1*	rs2241880	2	G	non-synonymous coding
	IRGM	rs10065172	5	T	exonic, synonymous coding
		rs4958847		A	intronic
	ULK1	rs12303764	12	T	
	LRRK2	rs11175593		T	
	MTMR3	rs2412973	22	A	downstream of gene
ER stress	*ORMDL3*	rs2872507	17	A	upstream of gene
	XBP1	rs35873774	22	C	intronic

ATG16L1: Autophagy Related 16 Like 1; IRGM: Immunity Related GTPase M; ULK1: Unc-51 Like Autophagy Activating Kinase 1; LRRK2: Leucine Rich Repeat Kinase 2; MTMR3: Myotubularin Related Protein 3; ORMDL3: ORMDL Sphingolipid Biosynthesis Regulator 3; XBP1: X-Box Binding Protein 1.

Ethical approval was given by the Ethics Board of the University Hospitals Leuven (B322201213950/S53684) and all patients provided written informed consent.

Table 1: Patient characteristics

# Patients	35
Female [%]	20 [57]
Median [IQR] age (yrs)	53 [42-57]
Median [IQR] age at diagnosis (yrs)	27 [21.5-36.5]
Median [IQR] disease duration (yrs)	20 [10.5-28.5]
UC/CD [%]	4/31 [11/89]
Prior abdominal surgery [%]	21 [60]
Smoking [%]	
Yes	12 [34]
Former	5 [14]
No	11 [31]
Unknown	7 [20]
Therapy [%]	
Antibiotics	2 [6]
Corticosteroids	2 [6]
Thiopurines/methotrexate	4 [11]
Anti-tumor necrosis factor	14 [40]
Vedolizumab	1 [3]

(IQR: interquartile range; yrs: years)

3.2. Isolation and culturing of IECs

The crypt isolation protocol and cell culture medium were adapted from the colonic organoid culture procedure which was developed by Sato *et al.*in 2011[34]. Biopsies were immediately placed in DMEM-F12 (Lonza, Basel, Switserland) containing glutamine (15 mM), hepes (15 mM) and penicillin/streptomycin (100 U/ml, Lonza, Basel, Switserland) at 4 °C for transport (on ice) to the research lab and stored (at 4 °C) for up to two hours until epithelial isolation was performed. First, the biopsies were washed in DMEM-F12 after which they were allowed to settle and the supernatant was discarded. Next, they were thoroughly washed with complete chelating solution (CCS, 0.996 g/l Na_2HPO_4*$2H_2O$, 1.08 g/l KH_2PO_4, 5.6 g/l NaCl, 0.12 g/l KCl, 15 g/l Saccharose, 10 g/l D-sorbitol, 80 mg/l DTT) by repeated pipetting. Finally, EDTA (10 mM, Thermo Scientific, Waltham, Massachusetts, USA) was added and the biopsy suspension was placed on a rocking platform at 4 °C for 45 minutes after which the EDTA containing solution was removed. The biopsy fragments were passed multiple times through a 10 ml pipette in CCS to mechanically disrupt the IECs from the remaining mucosal tissue leaving them in suspension as the remaining fragments settled down. The supernatant was

centrifuged after which the cell pellet was washed one more time in DMEM-F12 before resuspending the IECs in expansion medium (composition see **Supplementary table 1**) and plating the cells in collagen coated 12-well plates (7 wells/8 biopsies) in a humidified incubator at (37 °C, 5% CO_2). The medium was replaced for the first time after 24 hours and every 48 hours thereafter.

3.3. Immunocytochemistry

To further characterize these intestinal biopsy-derived cell cultures, several immunocytochemic stainings were performed. Isolated crypts were seeded on collagen coated CC2 Lab-Tek chamber well slides (Thermo Scientific, Waltham, Massachusetts, USA). At day 4, cells were washed with PBS and subsequently fixated in paraformaldehyde (PFA, 4%, 20', 37 °C)cells were washed again in PBS and permeabilized with methanol (10', RT). After washing, the cells were incubated in glycine (0.1 M, 2x10', RT) followed by washing and blocking (10 % FBS, 1% BSA, 1h, RT). The primary anti-E-cadherin antibody (ab1416, Abcam, Cambridge, UK) and the anti-platelet derived growth factor receptor-α (PDGFR-α) antibody (sc-338, Santa Cruz Biotechnology, Dallas, Texas, USA) were diluted in 1% BSA and cells were exposed for 1 hour. After washing in 1% BSA, the cells were incubated with the secondary goat anti-mouse antibody (Alexa Fluor 488, Thermo Scientific, Waltham, Massachusetts, USA) and goat anti-rabbit antibody (Alexa Fluor 594, Thermo Scientific, Waltham, Massachusetts, USA) for 1 hour followed by DAPI staining (1 µg/ml). After a final wash step in PBS-T, the cells were mounted with ProLong gold anti fade reagent (Life Technologies, Carlsbad, California, USA). Images were obtained using a BX41 microscope (Olympus, Tokio, Japan) and analyzed with the Scan R software (Olympus, Tokio, Japan). For the immunocytochemic staining of ZO-1, KI67 and MUC2 we used a slightly different protocol provided by the Gastrointestinal Motility and Sensitivity Research Group from KU Leuven. Washed cells (on Lab-Tek chamber slides) were fixated in PFA (4%, 30', RT) and rehydrated in 100% (3x3') and 70% (1x3') ethanol followed by H_2O (2x3'). Antigen retrieval was performed in sodium citrate buffer at 120 °C for 10' after which the cells were allowed to slowly cool to room temperature and were washed with PBS. Blocking was performed for 10' with Protein Block Serum-Free (Agilent, Santa Clara, CA, USA) for 10' after which they were incubated overnight at 4 °C with the primary antibodies: mouse-anti-ZO-1 (1/50, 339100, Thermo Scientific, Waltham, Massachusetts, USA), mouse anti-KI67 (1/150, MONX10283, Sanbio, Uden, The Netherlands), rabbit-anti-

MUC2 (1/150, sc-15334, Santa Cruz Biotechnology, Dallas, Texas, USA) diluted in Antibody Diluent (Agilent, Santa Clara, CA, USA). After washing in PBS, the cells were incubated with the secondary goat anti-mouse antibody (Alexa Fluor 488, Thermo Scientific, Waltham, Massachusetts, USA), donkey anti-rabbit antibody (Alexa Fluor 594, Thermo Scientific, Waltham, Massachusetts, USA) and DAPI (all 1/1000) for 30'. After washing, the cells were mounted with citifluor™ (VWR, Radnor, PA, USA). Normal fluorescent images were obtained with the a BX41 microscope (Olympus, Tokio, Japan) whereas a LSM 880 microscope with Airyscan (Zeiss, Oberkochen, Germany) was used to obtain high resolution z-stack images. Analysis was performed with ZEN Blue software (Zeiss, Oberkochen, Germany) and Fiji (ImageJ, NIH, Bethesda, MD, USA).

3.4. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Expression levels of epithelial marker genes cytokeratin-18 and -20 (*CK-18* and *CK-20*) were determined at 24, 72, 120 and 168 hours post isolation and compared with expression levels in the fetal human colon (FHC) cell line (positive control) and the IMR-90 lung fibroblast cell line (negative control). Furthermore, the expression of the intestinal epithelial stem cell marker *LGR5* was measured at the same time points. Finally baseline expression of the *GRP78/BIP* gene was measured in 6-day-old untreated IECs from 19/35 patients who were included in this study.

Cells were washed with PBS and mechanically removed in RNAlater using a cell scraper. After centrifugation the pellet was resuspended in RLT-buffer (Qiagen, Hilden, Germany) containing β-mercapto-ethanol and passed repeatedly through a 29G needle or Qiashredder tubes (Qiagen, Hilden, Germany). An equal volume of 70% ethanol was added and mRNA was extracted from this mixture with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. RNA quality was assessed with the NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) and samples were stored at -80 °C until cDNA synthesis with the Qscript cDNA supermix (Quantabio, Beverly, MA, USA) according to manufacturer's protocol.

The primers used for qRT-PCR analysis are given in **supplementary table 2** and 10 µM stock solutions were used to make the reaction mixture (5 µl SybrGreen, 0.2 µM FW & RV primer, 2.5 µl cDNA sample, 2.3 µl RNAse-free H_2O). Samples were analyzed with the Lightcycler 480

(Roche, Basel, Switzerland) and the following amplification program was used: 5' 95 °C, 45x (10" 95 °C, 15" 60 °C, 15" 72 °C), 5" 95°C, 1' 60 °C, 4 °C. *CK-18*, *CK-20*,*LGR5* and *BIP/GRP78* mRNA-levels were normalized to the housekeeping gene *β-actin* and quantified using the comparative (Δ or ΔΔ) C_t method.

3.5. ER stress induction

IEC cultures were treated for 14 hours (from the end of day 5 until the beginning of day 6) with the ER stress inducing compound thapsigargin (0.4 µM, Sigma-Aldrich, Saint Louis, Missouri, USA) in order to enhance potential inter-patient differences.Lysis and total protein measurement

After 14 hours of thapsigargin-treatment, the IECs were placed on ice, the medium was aspirated and the cells were rinsed with ice cold PBS. Next, the IECs were scraped in PBS and spun down, the resulting cell pellet was resuspended in RIPA lysis buffer (Enzo Life Sciences, Farmingdale, New York, USA) and lysis was performed by incubating the suspension 45' on ice followed by sonication. The cell lysate was spun down at maximal speed to pellet membrane fragments and the supernatant was used to determine the total protein content with the Pierce® BCA Protein Assay Kit (Thermo Scientific, Waltham, Massachusetts, USA), the remainder was stored at -80 °C until enough samples were acquired to perform an ELISA.

3.6. Binding immunoglobulin protein (BiP)/Glucose-regulated protein 78 (GRP78) ELISA

To quantify the ability of IECs to cope with ER stress, we measured BiP (also known as GRP78 or HSPA5) levels before and after thapsigargin-treatment with a competitive BiP ELISA kit (Enzo Life Sciences, Farmingdale, New York, USA) according to manufacturer's protocol. Plates were read with the Fluostar Omega microplate reader (BMG Labtech, Ortenberg, Germany) and quantified with a 5-PL logistic regression script in Microsoft Office Excell. ER stress induction rates were expressed as the BiP ratio between treated and untreated IECs ($[BiP]_{thapsigargin\text{-}treated}/[BiP]_{untreated}$).

3.7. Statistical analysis

As data were not normally distributed, non-parametric tests were used, no multiple testing was performed. The BiP (ELISA) levels and ratios were compared between the different groups using a Mann-Whitney test with Graphpad Prism Software (La Jolla, California, USA). A p-value < 0.05 was considered significant.

4. Results

4.1. IECs and epithelial characterization

After isolation, the intestinal crypts retained their three-dimensional morphology while being suspended in the medium (**Figure 1A**). During overnight incubation, the crypts sunk and attached to the collagen-coated surface to form an epithelial monolayer. These two-dimensional IEC-islands consisted of cuboidal cells, giving them a pavement-like appearance. Visually each IEC-island was formed around a growth center (**Figure 1B**, red arrow), suggesting these cells originated from proliferating intestinal stem cells originally located in the bottom of the crypts *in vivo*. Cells in this center divided and gradually pushed away earlier formed cells resembling the *in vivo* situation. These observations were confirmed by immunofluorescent stainings for the proliferation marker KI67 (**Figure 2**). Furthermore, as cells moved away from these areas of proliferation, a significant portion differentiated cells shows presence of MUC2-positive cells with a goblet cell-like morphology (**Figure 2**, separate fluorescence channel images are provided in **supplementary figure 2**).

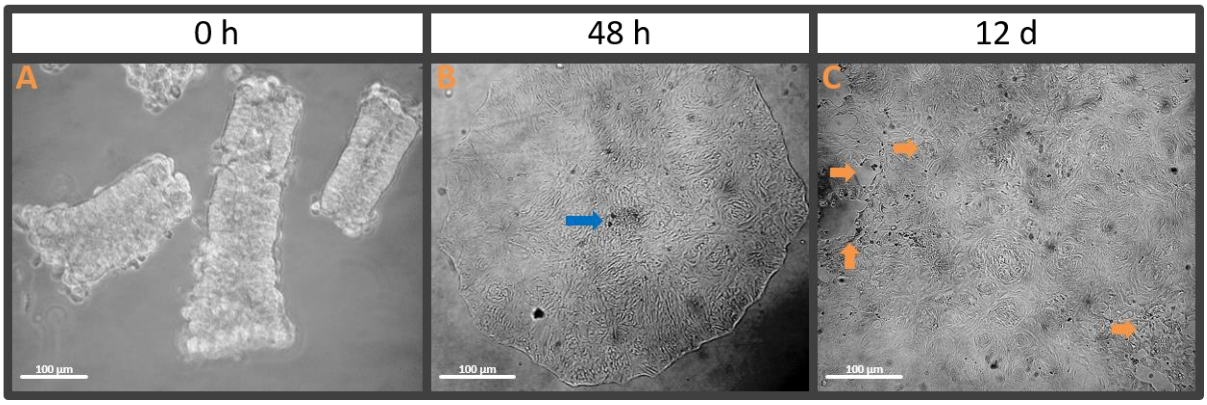


Figure 1: Brightfield (BF) microscopic image of freshly isolated colonic crypts with intact crypt architecture (A); BF image of an IEC-island with growth center (blue arrow) in a collagen coated well 48 hours post isolation (B); BF image of a 12-day-old IEC culture with typical areas of cell death and detachment (orange arrows) (C).

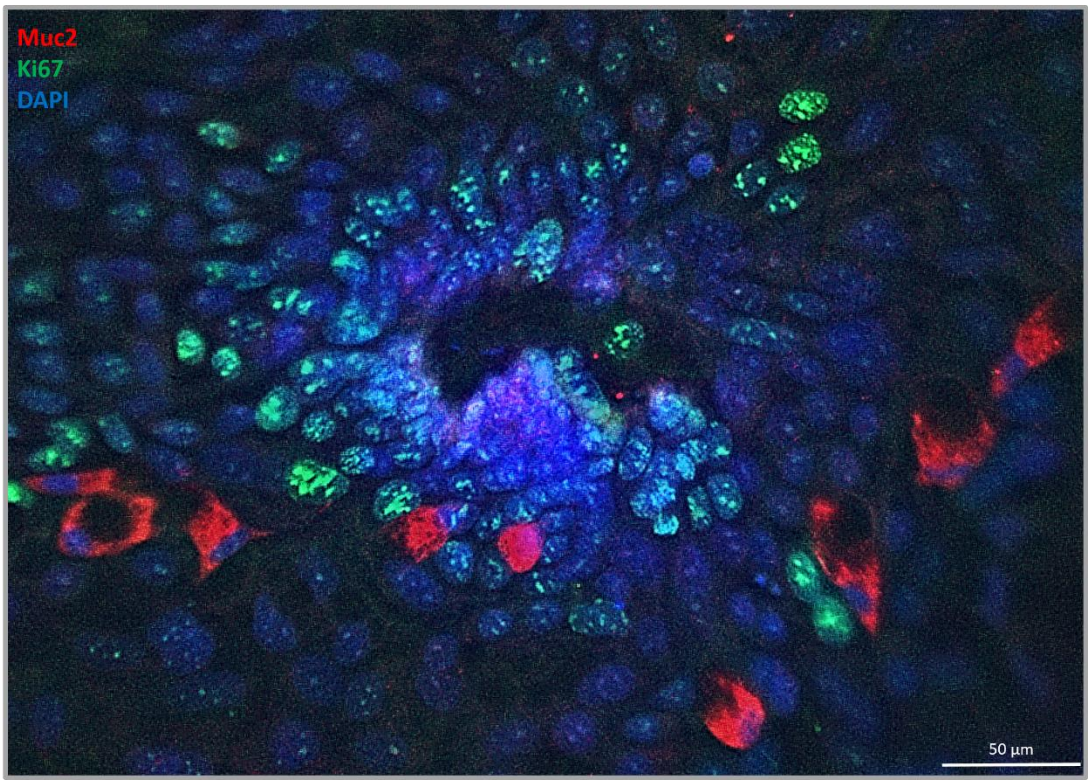


Figure 2: Immunocytofluorescent staining for Ki67 (green) and Muc2 (red) in a 4-day-old IEC culture (20x magnification, blue: DAPI staining).

Cells remained viable in culture for approximately 12 days, after which local cell detachment and cell death occurred (**Figure 1C**, red arrows). The combined isolation and culture success rate was 81% (39 out of 48 isolations). Failure was mostly due to low donor-dependent IEC isolation yields or inefficient cell attachment but was independent of presence of ER stress or autophagy risk alleles.

In order to confirm the epithelial character, 4-day-old IEC cultures were stained for E-cadherin [35, 36]. **Figure 3A** and **3B** illustrate how this epithelial transmembrane adherens junction protein was strongly expressed along the cell membranes of the cultured IECs. In order to rule out fibroblast contamination, we also performed a PDGFR-α staining which was negative in 4-days old IEC cultures. A positive control staining for this latter antibody on IMR-90 fibroblast cells is provided in **supplementary figure 3**.

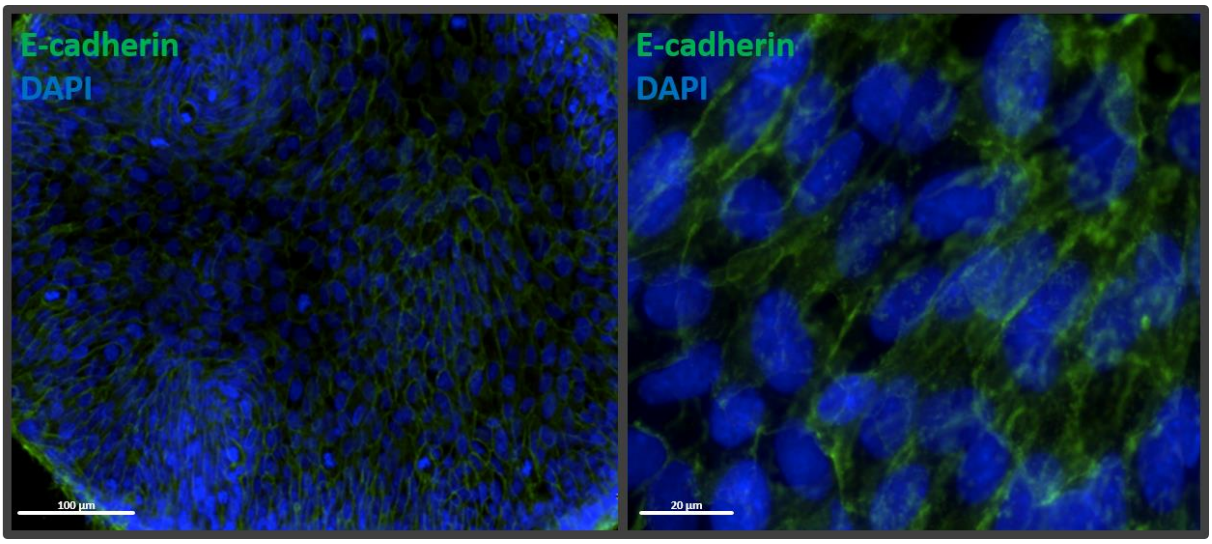


Figure 3: Immunocytofluorescent staining for E-cadherin (green) and PDGFR-α (red) in a 4-day-old IEC culture

The epithelial character of these *ex vivo* cell cultures was further assessed by measuring mRNA levels of *CK-18* and *CK-20* over time, as indicated in **figure 4**. In cultured IECs, *CK-18* was stably expressed over time (up to 168h), whereas *CK-18* mRNA could also be detected (in lower amounts) in FHCs but not in IMR-90 cells (**Figure 4A**). **Figure 4B** illustrates how *CK-20* is initially expressed at high levels and a gradually decreases over time. Still, also at day 7 (168 hpi), expression levels were strongly present when compared to proliferating cultures of FHCs. Additionally, we also measured the expression of the intestinal epithelial stem cell marker *LGR5* in IEC cultures at the same time points (24, 72, 120, 168 hpi) and showed that *LGR5* expression increased over time (**Figure 5**).

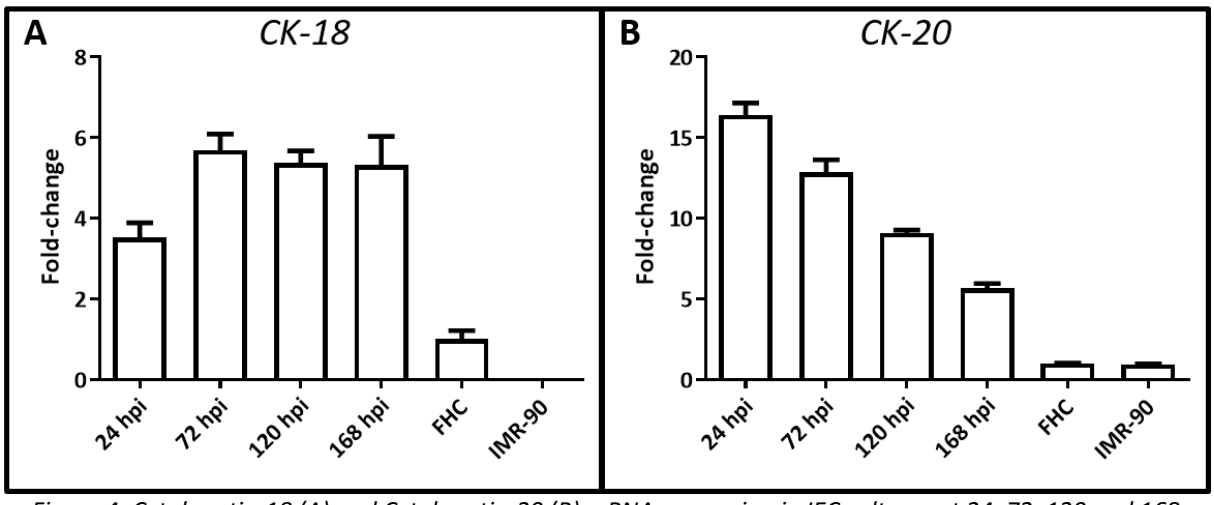


Figure 4: Cytokeratin-18 (A) and Cytokeratin-20 (B) mRNA expression in IEC cultures at 24, 72, 120 and 168 hours post isolation (hpi) and in the FHC and IMR-90 cell lines ($\Delta\Delta C_t$-method, fold change to expression levels in FHCs and all normalized to β-actin mRNA).

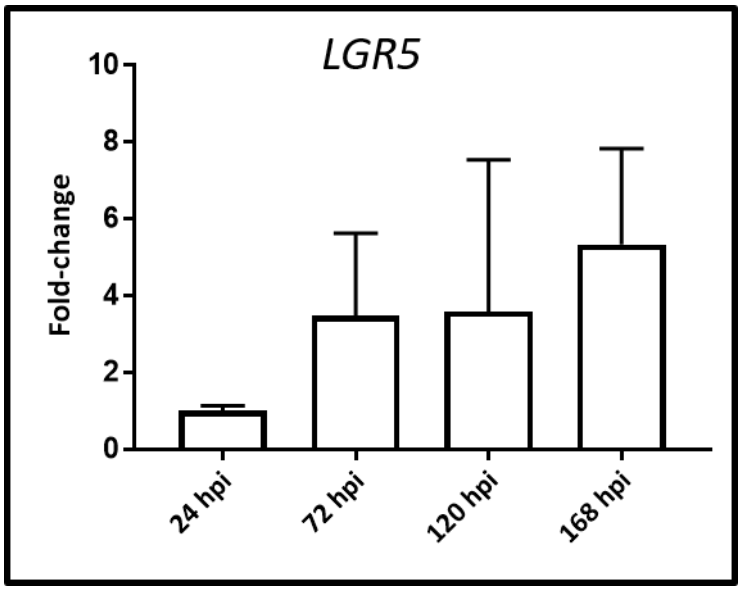


Figure 5: Lgr5 mRNA expression in IEC cultures at 24, 72, 120 and 168 hours post isolation (hpi). ($\Delta\Delta C_t$-method, fold change to expression levels at 24hpi and all normalized to β-actin mRNA).

Finally, we assessed the polarity along the apical-basolateral axis, by staining the cells for zonula occludens-1 (ZO-1). We could show that ZO-1-postive signal (green) is distributed apically at a depth of 0-1500 nm, whereas this positive signal disappears completely when moving closer towards the basolateral side (**Figure 6** and **7**).

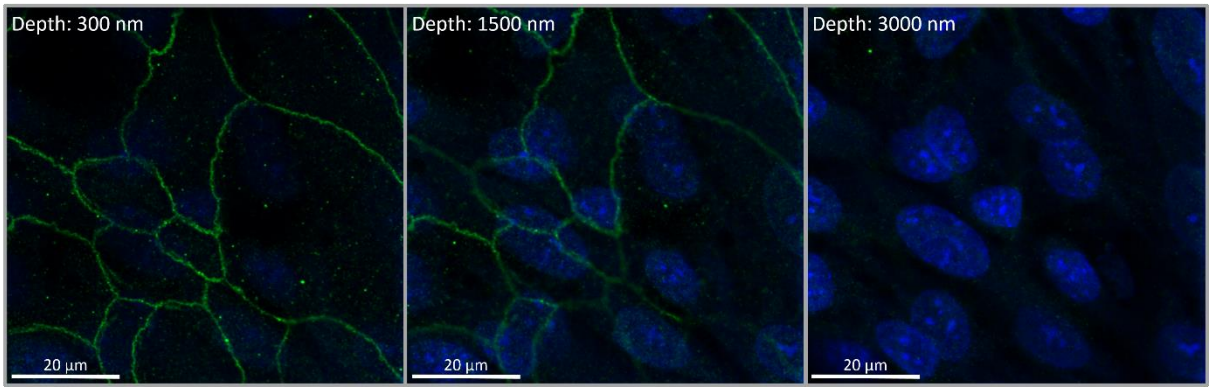


Figure 6: z-stack images at three different depths (300, 1500 and 3000 nm from the apical border) of a 4-day-old IEC culture stained for ZO-1 (green). (blue: DAPI staining)

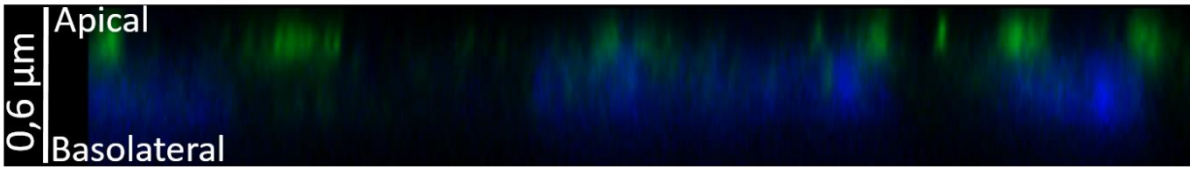


Figure 7: Cross sectional view of the reconstructed z-stack images from figure 6.

4.2. Genetic risk in ER stress and autophagy genes and the epithelial ER stress response

The IEC cultures were microscopically monitored daily between the time of isolation and lysis in order to exclude wells that had an aberrant morphology or showed signs of cell death. In

order to determine the cells' intrinsic capability to cope with ER stress, we measured BiP-levels with and without a 14 h treatment with the ER stressor thapsigargin from day 5 until day 6.

Patients were then grouped according to the number of ER stress risk alleles in *XBP1* (rs35873774) and *ORMDL3* (rs2872507) (**Table 1**). Median [IQR] thapsigargin-mediated BiP-induction was 2.67 [1.01-6.07], 1.87 [1.50-3.16], 1.70 [1.32-2.41] and 4.48 [3.76-4.64] in IECs from patients carrying 0, 1, 2 or 3 risk alleles respectively. Notice the absence of a group with patients carrying 4 ER stress risk alleles. Because of their low prevalence (0.14 %) in our patient genotype database (**Supplementary figure 1A**), we were unable to include these patients as this means that we had only 4 patients with 4 ER stress risk alleles in our entire genotyped patient cohort. These specific patients did not undergo an endoscopic investigated at our clinic during the time of inclusion. IECs from patients with three risk alleles had significantly more ER stress induction rates when compared to patients with one or with two risk alleles (**Figure 8A**, p=0.0262 and 0.0430, respectively).

We also grouped patients in risk quartiles, based on the number of risk alleles (RA) in autophagy genes *ATG16L1* (rs2241880), *IRGM* (rs10065172 and rs4958847), *MTMR3* (rs2412973), *LRRK2* (rs11175593) and *ULK1* (rs12303764). The distribution of these risk alleles in the sampled population (**Supplementary figure 1B**) was used to define the number of risk alleles in each quartile: Q1 had ≤3 RA, Q2 had 4 RA, Q3 had 5 RA and Q4 had ≥6 RA. Median [IQR] thapsigargin-mediated BiP-induction was 1.58 [1.13-2.85], 1.78 [1.52-2.64], 3.57 [1.83-4.64] and 2.74 [1.60-3.59] in IECs from patients belonging to Q1 to Q4 respectively (**Figure 8B**). No significant differences were observed between these groups (**Figure 3B**) although a trend towards higher ER stress induction rates in Q3 and Q4 compared to Q1 (p = 0.0507 and 0.1535, respectively) was seen.

Finally, given that autophagy and ER stress show a clear interplay[31, 32, 37, 38], ER stress and autophagy risk alleles were combined. This combination of risk alleles led to a change in the definition of the genetic risk quartiles (Q1: ≤4 RA, Q2: 5 RA, Q3: 6 RA, Q4: ≥7 RA; **Supplementary figure 1C**). Median thapsigargin-mediated BiP-induction [IQR] was 1.34 [1.08-1.91], 2.16 [1.68-4.05], 3.60 [1.39-4.48] and 2.41 [1.61-3.27] in IECs from patients belonging to genetic risk groups Q1 to Q4, respectively (**Figure 8C**). Patients in Q2, Q3 and Q4 had

significantly higher ER stress induction rates when compared to Q1 (p = 0.0343, 0.0401 and 0.0343, respectively).

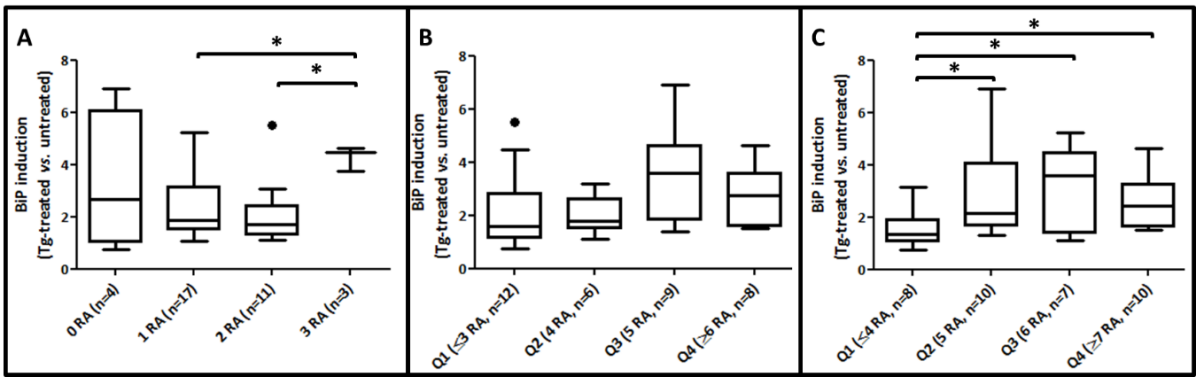


Figure 8: Boxplot diagrams showing the thapsigargin (Tg)-induced ER stress (BiP) levels of 6-day-old colonic IEC cultures from IBD patients carrying 0 to 3 ER stress-related risk alleles (n=4, 17, 11 and 3, respectively) in XBP1 and/or ORMDL3 (A); From IBD patients carrying ≤3 (Q1, n=12), 4 (Q2, n=6), 5 (Q3, n=9) or ≥6 (Q4, n=8) autophagy-related risk alleles in ATG16L1, MTMR3, ULK1 and/or LRRK2 (B); from IBD patients carrying ≤4 (Q1, n=8), 5 (Q2, n=10), 6 (Q3, n=7) or ≥7 (Q4, n=10) ER stress and autophagy-related risk alleles in XBP1, ORMDL3, ATG16L1, MTMR3, ULK1 and/or LRRK2 (C). (: Mann Whitney p-value < 0.05).*

At baseline, there were no significant differences in BiP mRNA or protein expression between the different patient subgroups (**Figure 9** and **10**, respectively).

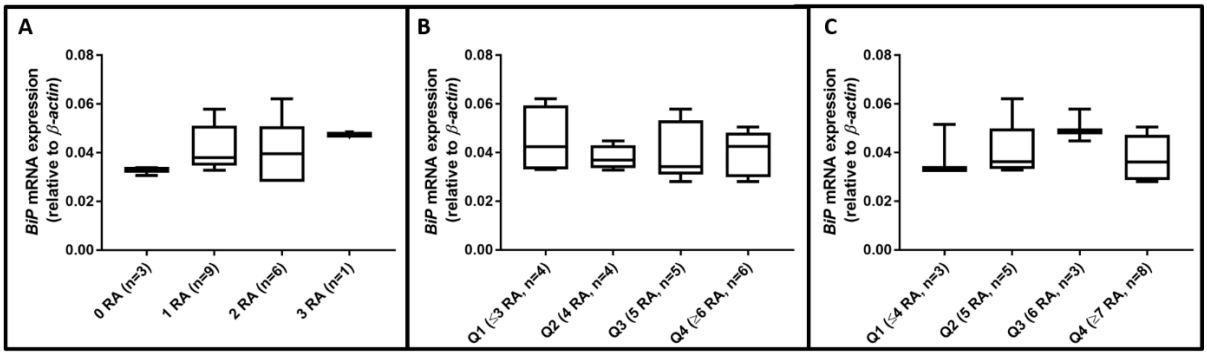


Figure 9: Boxplot diagrams showing the baseline ER stress (BiP) mRNA levels (normalized to β-actin mRNA) of 6-day-old colonic IEC cultures from IBD patients carrying 0 to 3 ER stress-related risk alleles (n=3, 9, 6 and 1, respectively in XBP1 and/or ORMDL3 (A); From IBD patients carrying ≤3 (Q1, n=4), 4 (Q2, n=4), 5 (Q3, n=5) or ≥6 (Q4, n=6) autophagy-related risk alleles in ATG16L1, MTMR3, ULK1 and/or LRRK2 (B); from IBD patients carrying ≤4 (Q1, n=3), 5 (Q2, n=5), 6 (Q3, n=3) or ≥7 (Q4, n=8) ER stress and autophagy-related risk alleles in XBP1, ORMDL3, ATG16L1, MTMR3, ULK1 and/or LRRK2 (C).

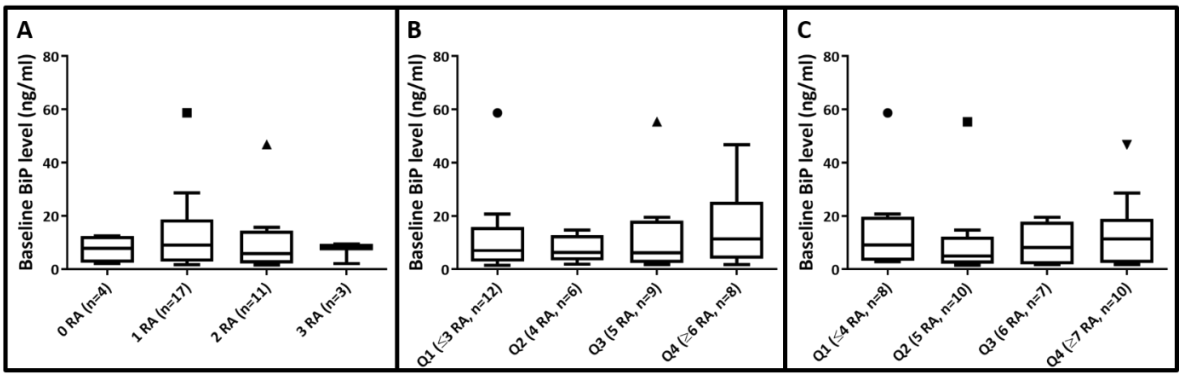


Figure 10: Boxplot diagrams showing the baseline ER stress (BiP) protein levels of 6-day-old colonic IEC cultures from IBD patients carrying 0 to 3 ER stress-related risk alleles (n=4, 17, 11 and 3 respectively) in XBP1 and/or ORMDL3 (A); From IBD patients carrying ≤3 (Q1, n=12), 4 (Q2, n=6), 5 (Q3, n=9) or ≥6 (Q4, n=8) autophagy-related risk alleles in ATG16L1, MTMR3, ULK1 and/or LRRK2 (B); from IBD patients carrying ≤4 (Q1, n=8), 5 (Q2, n=10), 6 (Q3, n=7) or ≥7 (Q4, n=10) ER stress and autophagy-related risk alleles in XBP1, ORMDL3, ATG16L1, MTMR3, ULK1 and/or LRRK2 (C).

5. Discussion

In this study we developed a novel *ex vivo* two-dimensional IEC culture model allowing characterization and quantification of pathogenic pathways in IBD in a patient-specific manner. We demonstrated that these biopsy-derived epithelial cell cultures remain viable for about 12 days and isolation success was more than 80%. The epithelial character was illustrated by a clear E-cadherin staining along the membranes of IECs, which resembles immunohistochemical E-cadherin stainings on human colonic tissue sections[39, 40]. We could not detect the fibroblast marker PDGFR-α which indicates that these cultures were free of contamination by mesenchymal cells. The areas where the crypts originally attached, remained a center of IEC proliferation as indicated by the abundance of KI67 positive cells. Daughter cells get pushed outward and either differentiate into intestinal epithelial cell types or retain their proliferative phenotype.

We also analyzed gene expression levels of two epithelial cytokeratins over time. Cytokeratin 18 is a type-1 keratin that is found in all simple epithelial tissues such as the intestinal epithelial lining and the proximal tubule of the kidney[41, 42]. We could detect stable *CK-18* mRNA levels, illustrating our monolayer cultures have an epithelial character that is not lost over time. Cytokeratin 20, on the other hand, also belongs to the type-1 keratin family and is predominantly expressed in differentiated IEC subtypes[41-43]. In our IEC cultures, *CK-20* mRNA levels were decreasing, suggesting loss of differentiation over time. This is further supported by the inverse correlation between the time-dependent *CK-20* and *LGR5* mRNA expression: *LGR5* expression increases over time indicating a rise in the relative abundance of epithelial stem cells.

Finally, since polarity is an important aspect of a functional epithelial monolayer, we stained the cells for zonula occludens-1 (ZO-1), a tight junction protein which should be located at the apical side of the epithelium. We could indeed show that ZO-1 is distributed apically when compared to the nuclei.

Taken together, these data confirm that the isolated cells form polarized epithelial monolayers that contain both proliferating and differentiated cells. This model therefore shows the potential for measuring specific biologic responses in individual patients stratified on genetic susceptibility, disease location and/or therapies.

As a further proof of concept, we also showed for the first time that the genetic susceptibility in two important pathways associated with IBD, namely ER stress and autophagy, can be functionally translated and quantified in individual patients using biopsy-derived IECs. We chose to focus on these two pathways because of their functional interaction and importance for IEC homeostasis[29-31]. We measured intracellular BiP-levels as a quantitative readout for the amount of ER stress. BiP or GRP78/HSPA5 is a molecular chaperone protein that is strongly involved in ER stress signaling. It is upregulated when ER stress increases (eg. after thapsigargin treatment) and controls further activation of all three branches of the unfolded protein response (the ER stress signaling pathway)[44, 45].

Two ER stress-related risk loci have been identified so far (rs35873774 and rs2872507) and patients carrying more than two risk alleles in this pathway were rare in our patient population. Therefore, it was impossible to further group patients into genetic risk quartiles. Hence, the highest risk group (carrying 3 RA) contained only three patients. Nevertheless, this patient-group showed a significant increase in thapsigargin-mediated ER stress (BiP) induction when compared to patients carrying two or one risk allele(s). These data illustrate a functional, quantifiable consequence of two confirmed genetic risk variants in the ER stress pathway in patients with IBD.

By clearing un- or misfolded intracellular proteins, autophagy by itself is an essential component of ER stress signaling[31, 37]. Accumulating evidence underscores the interaction of autophagy and ER stress signaling in the intestinal epithelium[31, 32, 38, 46]. For example, Adolph *et al.* showed in mice that epithelial specific genomic deletion of autophagy genes leads to increased ER stress signaling and vice versa. Both mechanisms thus seem to play compensatory roles in maintaining IEC homeostasis and preventing inflammation which is further demonstrated by the spontaneous ileitis that only occurs when both pathways are genetically perturbed[46]. Since it has been clearly demonstrated that dysfunctional autophagy also leads to increased ER stress in IECs[32, 38, 46, 47], we tried to confirm these murine findings using our human IEC model but were unable to detect significant differences in ER stress induction rates between patients belonging to different autophagy genetic risk quartiles. However, when ER stress and autophagy risk alleles were combined, a significant association between genetic risk and ER stress induction rates was seen. This indicates that the genetic risk in both pathways should be taken into account when looking at the functional level.

Finally, we could show that none of these patient subgroups showed significant baseline differences in the expression of BiP both at the mRNA and protein level. Therefore, these results suggest that it mainly the ability to cope with ER stress-inducing insult (eg. Thapsigargin) is affected rather than the baseline ER stress levels in stress-free conditions.

Our findings do not only show the functionality of this new *ex vivo* IEC culture system, they also demonstrate that disease-associated molecular pathways can be quantified in an individual patient. This may provide therapeutic opportunities such as the administration of ER stress reducing molecules in patients demonstrating increased ER stress levels in IECs. Despite the fact that ER stress is regarded a key player in the pathogenesis of IBD, it is currently not being considered as possible therapeutic strategy. Yet, the ER stress reducing conjugated bile acid tauroursodeoxycholic acid (TUDCA) may reduce epithelial apoptosis and inflammation and was shown to reduce severity of colitis in multiple IBD mouse models[15-18]. Furthermore, oral administration of TUDCA in the context of other diseases has not been associated with any adverse events so far [48-51]. It would therefore be very interesting to study if TUDCA could reduce inflammation in patients with IBD characterized by increased ER stress levels, as demonstrated in our human culture model. Besides TUDCA, other ER stress reducing compounds such as 4-phenylbutyrate (PBA) and glutamine could also be considered as these compounds also have shown some effectiveness in murine IBD models [13-15].

Likewise, the autophagic inducer rapamycin was effective in IBD case reports but failed to show efficacy in a randomized placebo controlled trial[9-12]. We wonder if functional characterization of the patients randomized in this study for defects in autophagy would shed a different light on the results.

Since these cells are grown in two dimensions, the apical side is easily accessible for pharmaceutical compounds or micro-organisms which is a great advantage compared to the 3-dimensional intestinal organoid model originally described by Sato et al[34]. Intestinal organoids are an excellent model to investigate multiple key aspects of intestinal epithelial physiology and pathologies such as epithelial stem cell proliferation studies. However, our *ex vivo* monolayer protocol may offer several practical advantages and an easier to use system for exposure studies.

This *ex vivo* IEC culture system may be used or modified for other applications than the investigation of IBD-associated genetic defects at the site of the intestinal epithelium. Epithelial defects in other diseases like celiac disease, post-infectious irritable bowel syndrome and intestinal cancer could be further elucidated and lead to more personalized therapeutic approaches. Another potential application of the *ex vivo* cell culture system is personalized drug toxicity screening assays. We are currently further modifying our protocol allowing the IECs to grow on transwell membranes in order to perform permeability assays. This setup could also be used for co-culturing IECs with other relevant intestinal cell types such as macrophages.

In summary, we have developed and characterized a 2-dimensional IEC culture system that allows easy exploration of patient-specific epithelial defects and/or responses. We could detect defects in epithelial ER stress handling in genetically predisposed patients and hereby show that this approach can be used for the detection and quantification of underlying pathogenic mechanisms. Personalized tools such as this will become highly valuable in complex disorders and will allow treatment of a defective pathway instead of a disease phenotype.

6. Supplementary material

Supplementary table 1: Composition of the IEC expansion medium.

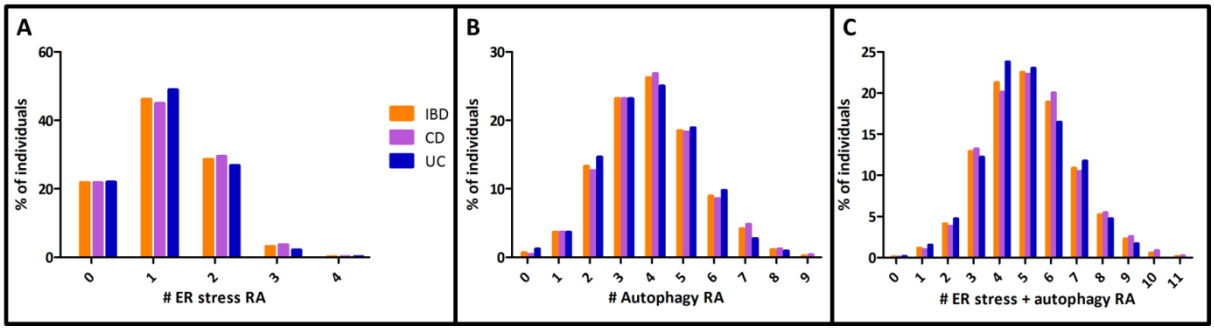
Compound	Final concentration	Source
Wnt3a	100 ng/ml	In house, cell line
Rspo1	100 ng/ml	
mNoggin	100 ng/ml	Peprotech, USA
Gastrin	10 nM	Sigma-Aldirch, USA
Nicotinamide	10 mM	
N-Acetylcysteine	1X	
SB202190	10 µM	
EGF	50 ng/ml	Invitrogen, USA
B27	1X	
N2	1X	
A83-01	500 mM	Tocris, UK

(Wnt3a: Wnt Family Member 3A; Rspo1: R-Spondin 1; mNoggin: murine Noggin; EGF: Epidermal Growth Factor)

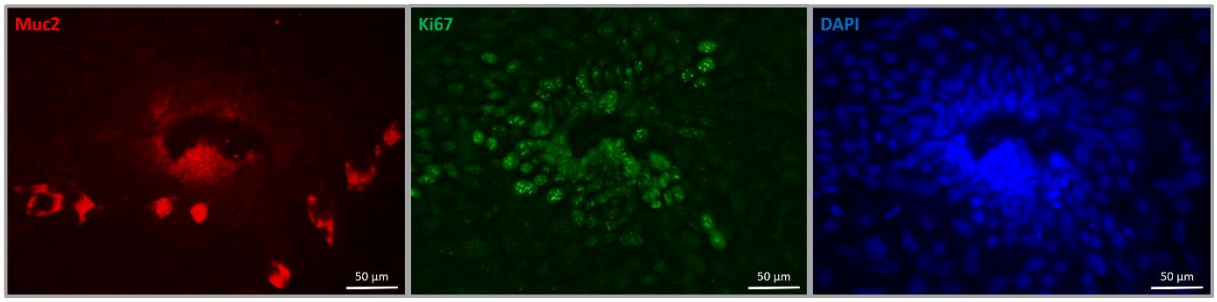
Supplementary table 2: Primers for qRT-PCR analysis.

Gene	Forward primer	Reverse primer
CK-18	5'-TGAGACGTACAGTCCAGT-3'	5'-GCTCCATCTGTAGGGCGT-3'
CK-20	5'-AGGAGACCAAGGCCCGTT-3'	5'-ATCAGTTGGGCCTCCAGA-3'
Lgr5	5'-ACCTCCTACCTAGACCTCAGT-3'	5'-CGCAAGACGTAACTCCTCCAG-3'
BiP/Grp78	5'-TGTTCAACCAATTATCAGCAAACTC-3'	5'-TTCTGCTGTATCCTCTTCACCAGT-3'
β-actin	5'-CCCAGCACAATGAAGATC-3'	5'-CTGATCCACATCTGCTGG-3'

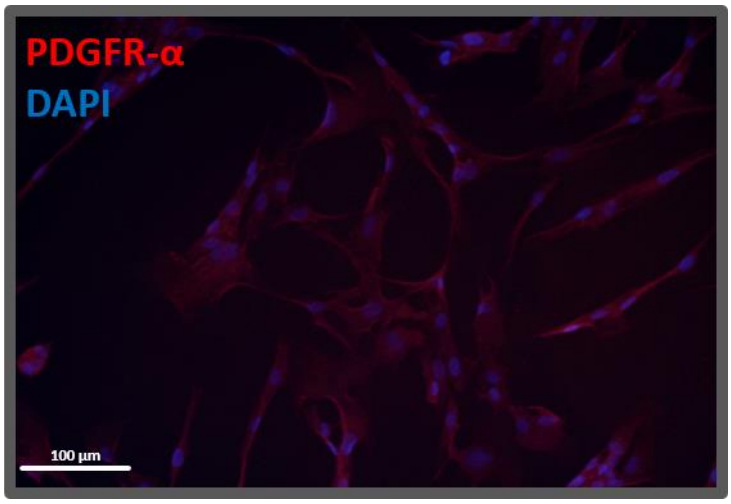
(CK: Cytokeratin; Lgr5: Leucine-rich repeat-containing G-protein coupled receptor 5; BiP: Binding immunoglobulin protein; GRP78: 78 KDa Glucose-Regulated Protein)



Supplementary figure 1: ER stress (A), autophagy (B) and combined (C) risk allele (RA) distribution in the immunochipped UC/CD/IBD patient population at the University Hospitals Leuven.



Supplementary figure 2: Separate channels of the immunocytofluorescent staining for Ki67 (green), Muc2 (red) in a 4-day-old IEC culture (Muc2: Mucin 2; DAPI: 4',6-diamidino-2-phenylindole).



Supplementary figure 3: Immunocytofluorescent staining for PDGFR-α (red) in the IMR-90 fibroblast cell line.

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CHAPTER 5

STRONG UPREGULATION OF AIM2 AND IFI16 INFLAMMASOMES IN THE MUCOSA OF PATIENTS WITH ACTIVE INFLAMMATORY BOWEL DISEASE

CHAPTER 5: Strong Upregulation of AIM2 and IFI16 Inflammasomes in the Mucosa of Patients with Active Inflammatory Bowel Disease

This entire chapter has been published as an original research article in Inflammatory Bowel Diseases: ***Vanhove W****, Peeters P M, Staelens D, Schraenen A, Van der Goten J, Cleynen I, De Schepper S, Van Lommel L, Reynaert N, Schuit F, Van Assche G, Ferrante M, De Hertogh G, Wouters E, Rutgeerts P, Vermeire S, Nys K, Arijs I. Strong upregulation of AIM2 and IFI16 inflammasomes in the mucosa of patients with active inflammatory bowel disease. Inflammatory Bowel Disease. 2015 Nov;21(11):2673-2682.*

1. Abstract

Background: Inflammatory bowel disease (IBD) is characterized by a chronic inflammation of the gut, partly driven by defects in the innate immune system. Considering the central role of inflammasome signaling in innate immunity, we studied inflammasome components in IBD mucosa.

Methods: Expression of genes encoding inflammasome sensor subunits was investigated in colonic mucosal biopsies from two cohorts of IBD patients and controls.

Results: A significant upregulation (>2-fold change in expression, false discovery rate <0.05) of the PYHIN inflammasomes *AIM2* and *IFI16*, in active IBD versus controls was found. Also *IFI16* was significantly increased in inactive IBD versus controls. Moreover, responders to anti-TNF therapy showed decreased expression of these inflammasomes, although *IFI16* remained significantly increased in responders showing endoscopic healing versus controls. AIM2 was mainly expressed in epithelial cells, whereas IFI16 was expressed in both lymphocytes and epithelial cells. Functional activation of predominant AIM2/IFI16-mediated inflammasomes in active IBD colon was shown by presence of the downstream effectors CASP1 and HMGB1, in inflamed mucosa.

Conclusions: Our results highlight the importance of PYHIN inflammasome signaling in IBD, and also link anti-TNF responsiveness to inflammasome signaling. Together, this points to the potential value of the inflammasome pathway as new therapeutic target for IBD treatment.

2. Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic debilitating inflammatory bowel diseases (IBD) with increasing prevalence worldwide[1]. IBD mostly affects young people and often leads to a greatly decreased quality of life, with diarrhea and abdominal pain as major gastrointestinal symptoms. Anti-TNF therapy has become the mainstay of therapy in IBD patients refractory to corticosteroids and/or immunomodulators, and is the first efficacious biological therapy for IBD[2].

While the exact pathogenesis of IBD is unknown, there is accumulating evidence of a genetic predisposition associated with bacterial sensing, autophagy, and links between the local microbial community and the mucosal immune system[3]. A balanced and tightly regulated innate intestinal immune response is important for intestinal homeostasis[4], preservation of an intact intestinal barrier function[5], and the elimination of invading pathogens[4]. Maintenance of cellular integrity and control of inflammation are indeed primordial for the host's survival and fitness. In the gut, the pro-inflammatory cytokines IL-1β and IL-18, together with TNFα, IL-8, IL-6 and others, are potent alarm signals that are involved in beneficial repair mechanisms and bacterial elimination. However, under abnormal chronic inflammation, they become detrimental and fuel chronic inflammation as well as remodeling[6-8].

IL-1β and IL-18 are released from the cell upon activation of inflammasomes. These are multi-domain protein complexes that assemble upon recognition of pathogen- or damage associated molecular patterns (PAMPs or DAMPs respectively) to comprise a platform for the primordial activation of inflammatory caspase-1 (CASP1)[9, 10]. Inflammasomes are characterized by their tripartite architecture: a sensor protein, an adaptor molecule (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) (ASC)), and an inactive enzyme precursor pro-caspase-1, that will be activated by proteolytic cleavage, and induce release of IL-1β, IL-18, alarmins (e.g. HMGB1) and growth factors[6, 9, 10]. Sensors can be classified as members of the NOD like receptor (NLR) protein family (NLRP1, NLRP2, NLRP3, NLRP6, NLRP7, NLRC4 and potentially NLRP12) or the pyrin and HIN domain-containing (PYHIN) protein family [myeloid cell nuclear differentiation antigen (MNDA), interferon-inducible protein X (IFIX, also known as PYHIN1), absent in melanoma (AIM2) and interferon inducible 16 (IFI16)][9, 10].

Genetic contributions to IBD have been intensively studied in the last two decades, leading to the discovery of 163 IBD-susceptibility loci[11]. The nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) gene shows the strongest association with CD and pointed towards the importance of the innate immune system in triggering onset of disease. Interestingly, also mutations in the *NLRP3* gene have been associated with CD while *NLRP2* and *NLRP7* mutations are associated with both CD and UC[12]. The role of inflammasomes in IBD seems to be ambiguous and highly dependent on the cell type. For example, mutations leading to a hypoactive inflammasome signaling can, in epithelial cells, have aggravating effects on IBD development in animal models. The opposite is thought to be true for inflammasome activation in the intestinal lamina propria (LP) where immunological cells such as macrophages and dendritic cells reside (*e.g.* by microbes that breach the intestinal barrier)[6].

Considering the central role of inflammasome signaling in innate immunity and the strong association of defects in the innate immune system with IBD, we investigated the presence and the functional activation of inflammasomes in mucosal biopsies of IBD patients with active or inactive disease compared to control individuals. In addition, we investigated the effect of anti-TNF therapy by analyzing expression patterns of these inflammasome components before and after control of inflammation.

3. Materials and methods

3.1. Patients and biopsy specimens

Cohort 1 (see **Supplementary table 1** for characteristics) contains colonic mucosal biopsies from 97 UC, 8 CD patients and 11 controls with normal mucosa. Biopsies were taken from the edge of the ulcers in the most inflamed part of the colon (sigmoid or rectum). Disease activity was endoscopically assessed. In UC, there were 74 patients with active disease (endoscopic Mayo subscore 2-3) and 23 with inactive disease (endoscopic Mayo subscore 0-1)[13]. In CD, all 8 patients had active disease (presence of ulcers). All controls underwent endoscopy for screening of polyps and had an endoscopically normal mucosa. For cohort 2, we used the colonic mucosal biopsies obtained from patients that were previously described by Arijs *et al.*[14]. This cohort included endoscopically-derived colonic mucosal biopsies from 43 refractory IBD patients with active colonic disease (24 UC, endoscopic Mayo subscore 2-3 and 19 CD, ulcers present) before and 4-6 weeks after their first anti-TNF [infliximab (Remicade; Centocor, Inc., Malvern, PA, USA)] infusion and from 12 controls with normal mucosa who underwent endoscopy for screening of polyps.

For both cohorts, half of the biopsies were immediately snap-frozen in liquid nitrogen and stored at -80°C for RNA isolation. The residual biopsies were used for routine histopathological examination.

3.2. Mucosal gene expression analysis

The mRNA expression of genes encoding members of the inflammasome complex that are discussed in the recent review of Aguilera *et al.* (*NLRP1*, *NLRP3*, *NLRP6*, *NLRP12*, *NLRC4*, *AIM2*, *IFI16*, *MNDA* and *PYHIN1*)[6] was investigated in endoscopic-derived colonic mucosal biopsies from the two cohorts, with the use of Affymetrix microarrays and quantitative RT-PCR (qRT-PCR).

3.3. RNA isolation

For both cohorts, total RNA was isolated from the biopsies with the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Total RNA quantity and quality was assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the 2100 Bioanalyzer (Agilent, Waldbronn, Germany), respectively. The extracted RNA was used for microarray and qRT-PCR analysis.

3.4. Whole-genome gene expression analysis

For cohort 1, total RNA (150 ng) was used to analyze gene expression via Affymetrix GeneChip® Human Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA), which are comprised of 33252 gene probe sets covering 99% of all well-annotated human genes. All steps were performed according to manufacturer's manual 4475209 Rev.B (Applied Biosystems, CA, USA). Protocol details are described in the online supplementary material.

The microarray data from cohort 1 were deposited according to minimum information about a microarray experiment (MIAME) guidelines to the Gene Expression Omnibus database. The cohort 1 array data was analyzed in R (http://www.r-project.org/). The raw data (.cel files) were pre-processed with robust multichip analysis to obtain a log2 expression value for each gene probe set using the implementation in the aroma.affymetrix R package[15]. For comparative analysis, linear models for microarray data (LIMMA)[16] was performed for all the gene probe sets present on the microarray to identify gene probe sets that are differentially expressed between the studied groups in cohort 1, based on moderated t-statistics with Benjamini-Hochberg false discovery rate (FDR) correction (adjusted p-value)[17].

To analyze gene expression in cohort 2 biopsies, total RNA was used to hybridise to Affymetrix Human Genome U133 Plus 2.0 arrays, which are comprised of 54675 probe sets representing most genes of the human genome according to manufacturer's manual as described in[14]. The cohort 2 microarray data have been submitted in MIAME format to the Gene Expression Omnibus (series accession number GSE16879). The cohort 2 raw data (.cel files) were also analyzed with Bioconductor tools in R. The robust multichip average method was performed on the Affymetrix raw data to obtain a log2 expression value for each probe set[18]. For pair-wise comparisons, LIMMA was performed for all probe sets present on the microarray to identify probe sets that are different between the studied groups in cohort 2, with Benjamini and Hochberg FDR correction.

For both cohorts, probe sets with FDR < 0.05 were considered significant.

3.5. qRT-PCR analysis

For validating the microarray data, qRT-PCR was performed for *AIM2* and *IFI16* on the RNA samples from both cohorts. β-actin was used as the endogenous reference gene. The primer

and probe sequences are given in **supplementary table 2**. Protocol details are described in the online supplementary material.

The relative target mRNA expression levels were calculated as a ratio relative to the β-actin reference mRNA[19]. Results were analyzed with SPSS software (SPSS, Chicago, IL, USA), using Mann-Whitney *U*-test for unpaired samples and Wilcoxon signed-rank test for paired samples. A p-value < 0.01 was considered significant.

3.6. Immunohistochemistry

To localize AIM2, IFI16, CASP1 and HMGB1 in the colonic mucosa, immunohistochemistry was performed on 5 µm-thick sections from formalin-fixed, paraffin-embedded endoscopic colonic biopsies from UC patients with active disease and control individuals (with n=3 in each group; patients were randomly selected from cohort 1).

Protocol details are described in the online supplementary material.

3.7. Western blot analysis

To detect IFI16 and AIM2 protein in mucosal tissue homogenate from fresh-frozen UC patient and control biopsies, the techniques were used as described in Arijs *et al*. 2011[14]. Densitometric analysis of AIM2 and IFI16 protein expression was performed by using the ImageJ software (National Institute of Health, Maryland, USA). The generated data were normalized to β-actin expression in order to obtain relative expression readouts. Anti-β-actin, anti-AIM2 and anti-IFI16 antibodies (mouse IgG) were obtained from Sigma-Aldrich, the secondary HRP-conjugated anti-mouse antibodies were purchased from Thermo Fisher Scientific.

3.8. Ethical considerations

The study was carried out at the University Hospital Gasthuisberg in Leuven, Belgium. Informed consent was obtained from all participants and the study was approved by the Ethics Board of the University Hospital Leuven (B322201213950/S53684).

4. Results

4.1. Gene expression of different inflammasome subtypes in IBD colonic mucosa

In cohort 1, the nine inflammasome genes (*NLRP1*, *NLRP3*, *NLRP6*, *NLRP12*, *NLRC4*, *AIM2*, *IFI16*, *MNDA* and *PYHIN1*) were represented by nine gene probe sets on the Affymetrix GeneChip® Human Gene 1.0 ST array (**Figure 1A** and **Table 1**). In cohort 2, 17 probe sets on the Affymetrix Human Genome U133 Plus 2.0 arrays represented the nine inflammasome genes (**Figure 1B** and **Table 2**).

First, we investigated the differential gene expression of the inflammasome sensor subunits in (non-)inflamed colonic mucosa of patients with (in-)active IBD and controls (cohort 1; **Figure 1A**; **Table 1**). The expression of all nine inflammasome genes, except *NLRP6*, was significantly upregulated in inflamed colon of active IBD patients when compared to non-inflamed colon of controls and inactive IBD patients. The increase of the colonic expression levels of the PYHIN inflammasomes *AIM2*, *IFI16* and *MNDA* was more pronounced (>2-fold) than NLR inflammasome genes (<2-fold). Furthermore, *IFI16* colonic gene expression was, although to a lesser extent, also increased in inactive IBD patients versus control colons. No significant difference in expression of the inflammasome genes was observed in inflamed colon between UC and CD.

Table 1: Fold changes of the gene probe sets (GeneChip® Human Gene 1.0 ST arrays) encoding the inflammasome genes from the comparative analyses performed in cohort 1 for UC, CD and IBD.

gene probe set ID	gene Symbol	active versus control colons (n=11)			inactive versus control colons (n=11)			active (A) versus inactive (IA)		
		UC (n=74)	CD (n=8)	IBD (n=82)	UC (n=23)	CD (n=0)	IBD (n=23)	UC (n_A=74, n_{IA}=23)	CD (n_A=8, n_{IA}=0)	IBD (n_A=82, n_{IA}=23)
7921434	*AIM2*	2,61**	2,03*	2,55**	1,15	NA	1,15	2,27**	NA	2,22**
7906400	*IFI16*	3,47**	2,64*	3,38**	1,69*	NA	1,69*	2,05**	NA	2,00**
7906377	*MNDA*	2.97**	2.96*	2.97**	1,00	NA	1,00	2.98**	NA	2.98**
8051396	*NLRC4*	1,44*	1,45	1,44*	0,90	NA	0,90	1,59**	NA	1,59**
8011884	*NLRP1*	1,57**	1,43*	1,56**	1,07	NA	1,07	1,46**	NA	1,45**
7911178	*NLRP3*	1,88*	1,57*	1,85*	1,10	NA	1,10	1,72**	NA	1,68**
7937305	*NLRP6*	1,02	1,00	1,02	0,93	NA	0,93	1,10*	NA	1,10*
8039096	*NLRP12*	1,45*	1,41*	1,45*	1,04	NA	1,04	1,40**	NA	1,40**
7906386	*PYHIN1*	1.67**	1.56*	1.66**	1,10	NA	1,10	1.52**	NA	1.50**

**: FDR<0.05, **: FDR<0.001, NA: not applicable*

Table 2: Fold changes of the probe sets (Human Genome U133 Plus 2.0 arrays) encoding the inflammasome genes from the performed comparative analyses in cohort 2 for UC, CD and IBD.

probe set ID	gene symbol	before infliximab versus control colons (n=6)			R after infliximab versus control colons (n=6)			NR after infliximab versus control colons (n=6)			R after infliximab versus R before infliximab			NR after infliximab versus NR before infliximab		
		UC (n=24)	CD (n=19)	IBD (n=43)	UC (n=8)	CD (n=11)	IBD (n=19)	UC (n=16)	CD (n=7)	IBD (n=23)	UC (n=8)	CD (n=11)	IBD (n=19)	UC (n=16)	CD (n=7)	IBD (n=23)
206513_at	*AIM2*	6.22**	5,11**	5,70**	1,47	1,63	1,56	4,54**	6,39*	5,04**	0,39*	0,44*	0,42**	0,57	0,76	0,62
206332_s_at	*IFI16*	5.73**	4,93**	5,36**	2,11	2,15	2,14	5,74**	6,15*	5,86**	0,47	0,55	0,51*	0,89	0,84	0,87
208965_s_at	*IFI16*	8.75**	7,33**	8,09**	3,19	3,17*	3,18*	7,63**	8,73*	7,95**	0,48*	0,51	0,50*	0,76	0,85	0,78
208966_x_at	*IFI16*	5.91**	5,17**	5,57**	2,21	2,20	2,21	5,72**	6,48*	5,94**	0,49	0,54	0,52**	0,85	0,81	0,84
204959_at	*MNDA*	10.77**	7.59*	9.23**	1,10	1,14	1,12	7.04*	30.23**	10.97**	0.24*	0.33*	0.29**	0,43	0,99	0,55
1552553_a_at	*NLRC4*	1,30	1,24	1,27	0,87	0,92	0,90	1,16	1,28	1,19	0,77*	0,79	0,78**	0,83	0,91	0,86
1552554_a_at	*NLRC4*	0,99	0,96	0,98	1,09	0,98	1,03	0,91	0,97	0,92	1,08	1,04	1,05	0,93	0,96	0,94
210113_s_at	*NLRP1*	1.36*	1,23	1,30	1,10	1,07	1,08	1,28*	1,65*	1,38*	0,85	0,98	0,92	0,92	1,18	0,99
211822_s_at	*NLRP1*	1.29*	1,24*	1,27*	1,09	1,05	1,06	1,26	1,49	1,33	0,88	0,89	0,88	0,96	1,14	1,01
211824_x_at	*NLRP1*	1.31*	1,18	1,25	0,97	1,02	1,00	1,27	1,65	1,37	0,86	0,96	0,92	0,90	1,23	0,99
207075_at	*NLRP3*	1,78	1,50	1,65	1,06	1,05	1,06	1,51	2,86*	1,84	0,85	0,91	0,89	0,71	1,23	0,84
216015_s_at	*NLRP3*	1,11	0,99	1,06	0,95	0,93	0,93	1,00	1,13	1,04	0,88	1,02	0,96	0,89	1,00	0,92
216016_at	*NLRP3*	1,09	1,03	1,06	1,04	0,99	1,01	1,06	1,14	1,08	1,02	0,97	0,99	0,94	1,07	0,98
1552932_at	*NLRP6*	0,87	0.84*	0.85*	0,94	0,98	0,96	0,83	0,86	0,83	1,02	1,12	1,08	0,97	1,08	1,01
1554952_s_at	*NLRP12*	0,98	0,93	0,96	0,93	0,92	0,93	0,95	1,03	0,97	0,94	0,99	0,97	0,97	1,11	1,01
223944_at	*NLRP12*	0,87	0,91	0,88	0,89	0,88	0,88	0,88	1,06	0,93	0,99	0,99	0,99	1,03	1,15	1,06
240413_at	*PYHIN1*	1,38	1.31*	1,35	0,99	1,05	1,02	1,36	1,54	1,41	0,90	0,88	0,89	0,88	1,01	0,92

**: FDR<0.05, **: FDR<0.001, R: responders, NR: non-responders*

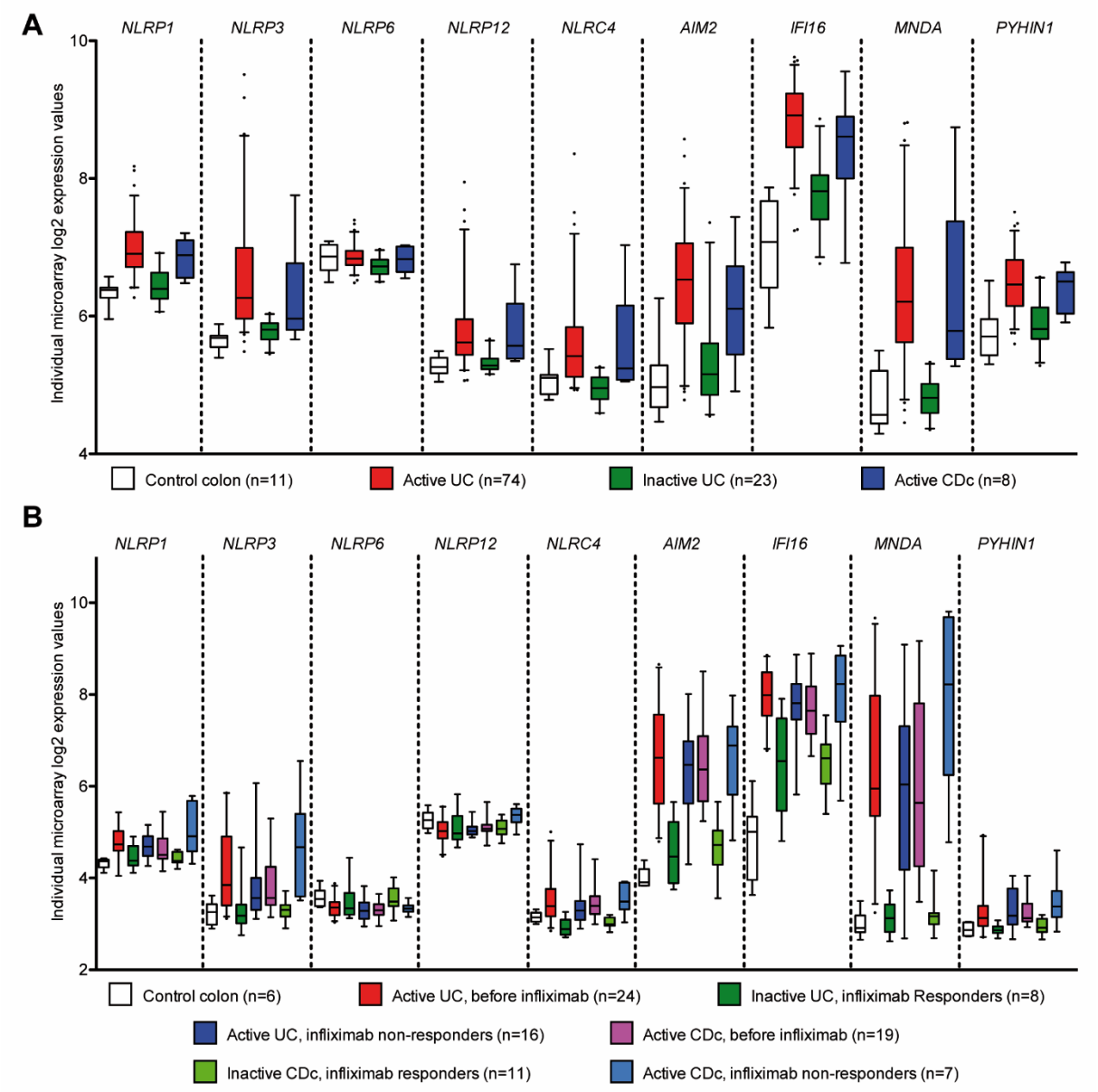


Figure 1: Box plots representing the microarray colonic expression of the 9 inflammasome genes of the different groups in cohort 1 (A) and cohort 2 (B).

The findings in cohort 1 were confirmed in cohort 2 (**Figure 1B** and **Table 2**). A significant increase in expression levels of *AIM2*, *IFI16*, *MNDA* and *NLRP1*, and a borderline significant increase ($FDR_{IBDvscontrols\_207075\_at}$= 0.08) of *NLRP3* expression in inflamed IBD colon *vs.* control colon was observed. Moreover, the colonic expression levels of *AIM2* (>5-fold), *IFI16* (>5-fold) and *MNDA* (>10-fold) were increased to a much greater extent than the colonic expression levels of *NLRP1* (~1,2-fold) and *NLRP3* (1.6-fold) in active IBD *vs.* controls. A significant decrease of *NLRP6* gene expression was seen in inflamed IBD colon *vs.* control colons.

Next, we studied the effect of anti-inflammatory treatment with infliximab on the expression of the inflammasome genes in inflamed colonic mucosa of active IBD (cohort 2; **Figure 1B**;

Table 2). After infliximab therapy, a significant decrease in expression of *AIM2*, *IFI16*, *MNDA* and *NLRC4* was observed in IBD responders showing complete colonic mucosal healing, when compared to their baseline samples. Although a decreased expression of *IFI16* was seen after infliximab therapy in IBD responders versus baseline samples, the *IFI16* expression levels remained significantly higher after infliximab therapy in IBD responders (= inactive IBD) versus control colons. In contrast with IBD responders, the colonic expression of *AIM2*, *IFI16*, *MNDA* and *NLRP1* remained significantly increased after infliximab therapy in IBD non-responders versus controls.

As the PYHIN inflammasomes showed the strongest and most significant upregulation in UC patients, for our further investigation we focused on the two most described PYHIN inflammasome sensor subtypes AIM2 and IFI16 in tissue from UC patients and controls. We confirmed the differential colonic gene expression of *AIM2* and *IFI16* observed by microarray analysis by qRT-PCR (**Supplementary figure 1** and **Supplementary table 2** and **3**).

4.2. Validation of AIM2 and IFI16 expression at the protein level

We next tested if AIM2 and IFI16 protein expression was also increased in colonic mucosa of active UC compared to controls on protein level, in randomly selected patients and controls from cohort 1. We confirmed the gene expression results, and found an increased colonic expression for both proteins in active UC mucosa versus normal control mucosa, although the increase of AIM2 protein was more pronounced. In the mucosa of patients with inactive UC, detected protein levels were intermediate between the levels of normal controls and patients with active disease, which is in accordance with our gene expression data. The readouts of the semi-quantitative densitometric analysis are represented in **Figure 2**. As the relative proportion of extracellular matrix protein versus intracellular protein can highly vary between biopsies and may severely affect total protein amount in the whole biopsy lysates, it is essential to normalize versus an intracellular housekeeping protein (such as β actin) in order to densitometrically quantify an intracellular protein. Pictures of the actual Western blot can be found in the supplementary material (**Supplementary figure 2 & 3**).

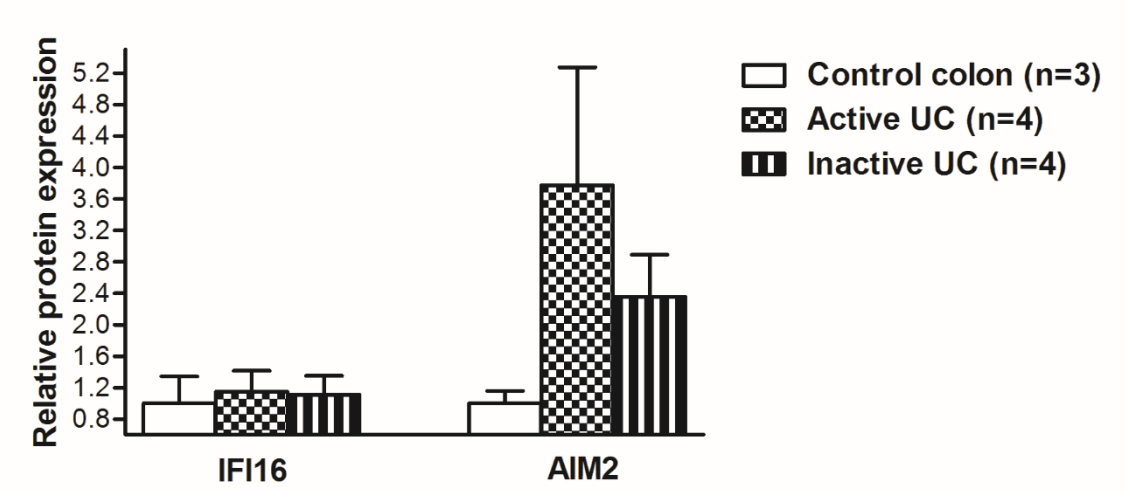


Figure 2: Semiquantitative densitometry analysis (ImageJ software) of the pooled AIM2 and IFI16 signal from Western blot on colonic biopsy lysates from control individuals and patients with active or inactive UC. AIM2/IFI16 expression levels were normalized to b-actin expression, and the relative expression in control colons was set to 1 (mean + SEM).

4.3. Immunohistochemistry

To evaluate whether AIM2 and IFI16 are expressed in distinct cell types of the mucosa, we localized these inflammasome sensor subunits by immunostaining of tissue sections from colonic biopsies from active UC patients and normal controls (**Figure 3**). These proteins demonstrate distinct localizations within the tissue but also within the cells. In normal colonic mucosa of healthy controls, AIM2 expression was seen throughout the epithelial cytoplasm, and not at the brush border (**Figure 3C**). In UC patients, the epithelial expression pattern of AIM2 was preserved and additionally some LP cells (**Figure 3D**, green arrow) and intra-epithelial lymphocytes (**Figure 3D**, red arrow) expressed AIM2. The other investigated sensor subunit, IFI16, showed a clear nuclear localization in LP cells in healthy controls as well as in active UC patients. However, in active UC colon, IFI16 showed stronger expression in all epithelial cells. This is in contrast to healthy controls where a gradient in expression could be seen, with clear IFI16 expression in the crypt base and a decrease in expression towards the apical side.

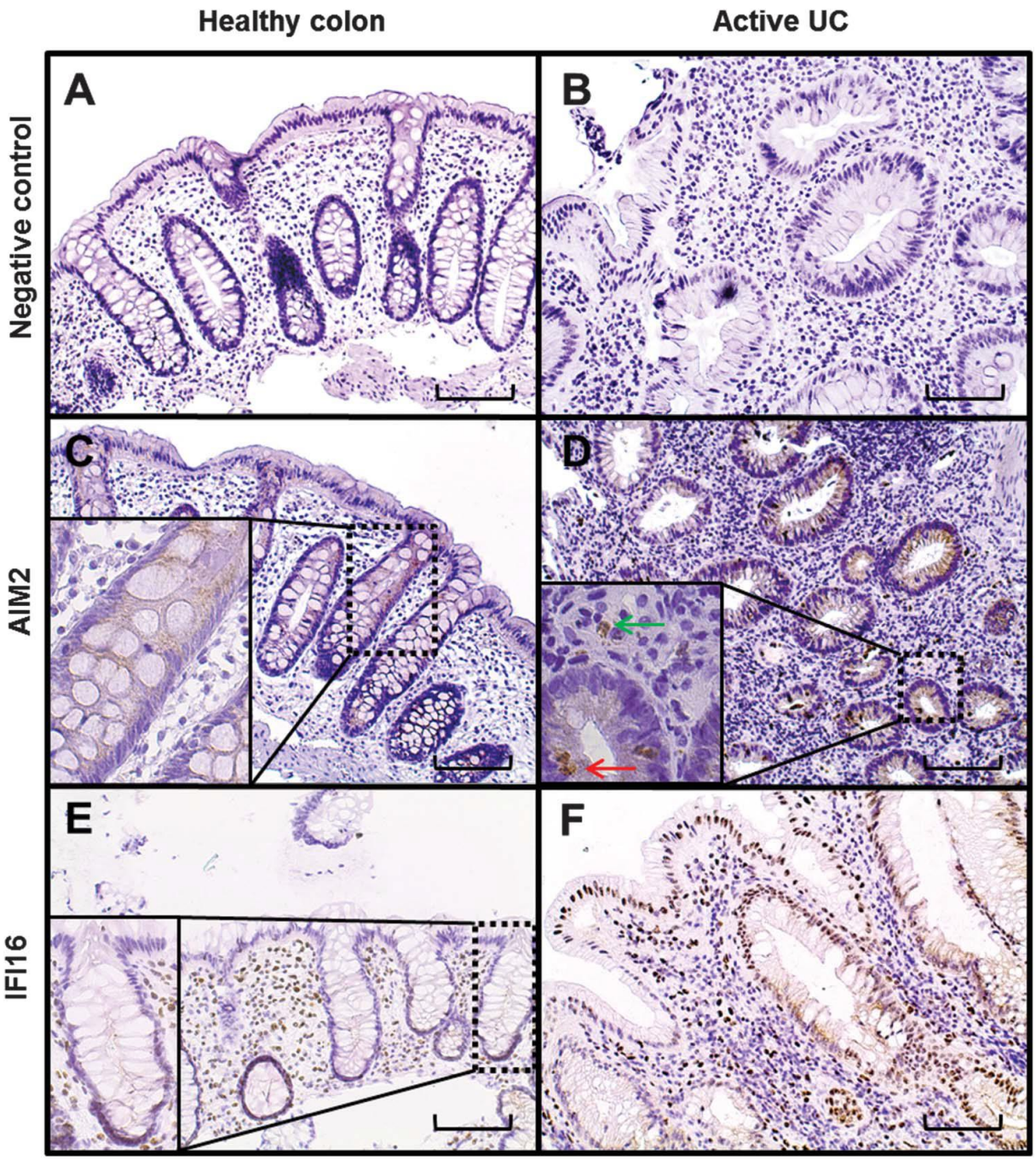


Figure 3: Immunohistochemistry for detection and localization of AIM2 (C and D) and IFI16 (E and F) in paraffin-embedded formalin-fixed tissue sections from colonic biopsies from control individuals (left) and patients with active UC (right). Negative controls with no primary antibody are shown in (A and B). Scale bars represent 100 µm, red arrow indicates an AIM2-positive LP cell, green arrow indicates an AIM2-positive intraepithelial lymphocyte.

Analogous to AIM2 and IFI16, we also analyzed the specific localization of two downstream effectors CASP1 and HMGB1 (**Figure 4**). CASP1 showed positive staining in the cytoplasm of epithelial cells but also in LP cells that are in proximity of the colonic epithelium of healthy controls. In UC patients, CASP1 was less abundant in the LP, but showed a high expression in the cytoplasm of epithelial cells from the crypt bases to the apical side. HMGB1 had a clear nuclear localization in a subset of LP cells, whereas in epithelial cells, this inflammasome

effector could be detected in both the nucleus and cytoplasm. This is in contrast with UC patients where HMGB1 was less abundant in the cytoplasm of epithelial cells, while nuclear expression remained the same. In the LP however, nuclear HMGB1 expression was generally increased.

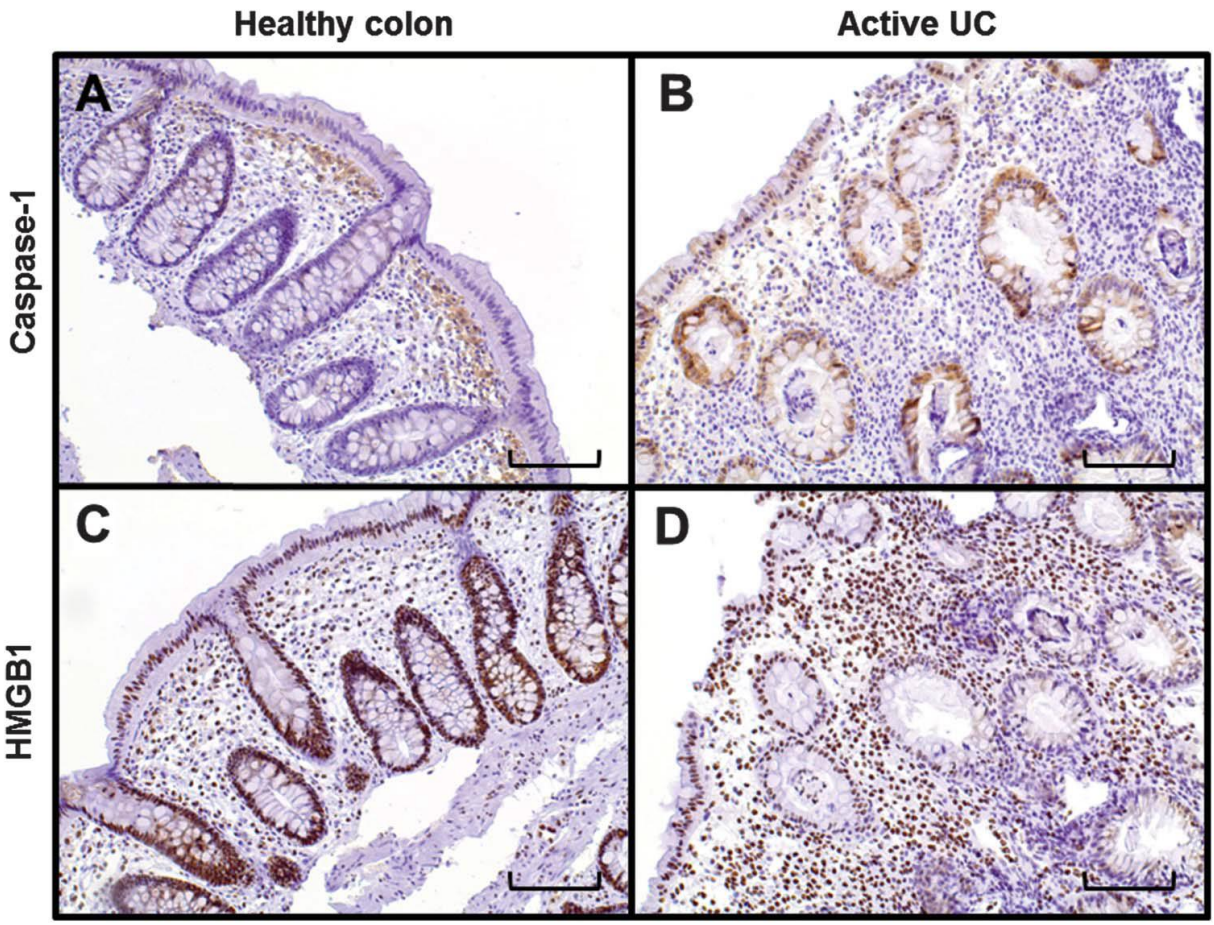


Figure 4: Immunohistochemistry for detection and localization of Caspase-1 (A and B) and HMGB-1 (C and D) in paraffin-embedded formalin-fixed tissue sections from colonic biopsies from control individuals (left) and patients with active UC (right). Scale bars represent 100 µm.

Our immunohistochemical data could confirm our gene expression results and indicate a marked presence of PYHIN (i.e. AIM2 and IFI16) inflammasome components in the epithelial compartment of the colonic mucosa in IBD patients with active disease.

5. Discussion

Current treatment options for IBD are far from successful in all patients[20]. Therefore, the identification of new pathways that contribute to the pathogenesis of IBD is essential in order to reveal new potential targets. We analyzed gene expression patterns and protein levels of inflammasome sensors in intestinal biopsies and compared these results between control individuals, (in-)active IBD patients and infliximab responding and non-responding patients.

We report the upregulation of most inflammasome sensor subtypes (*NLRP1*, *NLRP3, NLRP12*, *NLRC4*, *AIM2*, *IFI16*, *MNDA* and *PYHIN1*) in the colonic mucosa of active IBD patients, with the double stranded DNA (dsDNA) responding PYHIN inflammasome subtypes (*MNDA*, *AIM2* and *IFI16*) showing the strongest increase. Our immunohistochemical data show, besides inflammatory cell presence, an epithelial presence of AIM2 and IFI16 and some of their effector molecules (CASP1 and HMGB1). We have confirmed that dsDNA, which is a PYHIN/AIM2-IFI16-specific inflammasome trigger, can induce IL-1β maturation *in vitro* in primary intestinal epithelial cells (data not shown), suggesting AIM2 and/or IFI16-mediated inflammasome activation in this cell type.

The increased presence of dsDNA-responding inflammasomes could indicate an augmented mucosal dsDNA exposure for which several sources are possible, including mitochondrial DNA from damaged mitochondria or fragmented nuclear DNA from the decaying nucleus of the cell itself or from neighboring cells. Old/damaged mitochondria generate excessive reactive oxygen species (ROS) and may release their mitochondrial dsDNA in the cytosol[21]. While ROS could induce NLRP3 inflammasomes[22, 23], mitochondrial dsDNA has been shown to be able to act as a trigger of IFI16 & AIM2-inflammasomes. Additionally, mitochondrial inflammasome activation can be linked to defective autophagy, an event already associated with IBD[11, 24-28], and known to result in reduced clearance and increased accumulation of malfunctioning mitochondria and release of mtDNA[21] but also other disease related events such as decreased antigen sampling and IL-10 secretion by dendritic cells[29]. During chronic intestinal inflammation, there often is a strong imbalance between epithelial cell proliferation in the crypts and cell death at inflammatory hotspots. This may lead to epithelial barrier defects, an event which further promotes intestinal inflammation via an increased antigen exposure to the LP[30, 31]. Increased epithelial cell death results in fragmenting nuclear DNA, hereby potentially triggering PYHIN inflammasome activation.

Shimada *et al.* showed that oxidized mtDNA, which is formed and released during apoptosis, results in increased IL-1β secretion through NLRP3 inflammasome activation in macrophages[32]. The authors state that this response is probably a consequence of both NLRP3 activation as well as AIM2 activation by non-oxidized mtDNA[32]. Our findings indicate that this might also be the case for epithelial cells in the stressful environment of an active IBD mucosa.

Moreover, active CASP1 is a known inducer of pyroptosis[6], an inflammatory form of cell death believed to prevent spreading of intracellular pathogens such as *Lysteria monocytogenes,* which in the case of *Lysteria* infection requires both NLRP3 and AIM2-inflammasomes[33]. Inflammasome activation could thus by itself lead to the induction of cell death and inflammation as a feedback loop on intestinal inflammation.

Exogenous dsDNA sources are likely as well, for example from micro-organisms, bacteria or viruses that are able to invade the cell and release or produce dsDNA. Given the current hypothesis that IBD results from an aberrant immune response against commensal gut flora, and the higher concentrations of mucosal bacteria in IBD patients compared to healthy individuals[34], it is not surprising that pattern recognition receptors (PRRs), such as the IFI16 and AIM2-inflammasomes, are upregulated/stimulated in these patients. Moreover, shifts in bacterial composition or dysbiosis, a phenomenon associated with IBD[35, 36], might allow pathogens to invade the mucosa more easily (e.g. due to decreased nutrient competition with commensals or disturbed intestinal homeostasis)[37], which will consequently aid intestinal inflammation trough PRR stimulation. This suggests that commensal microbial DNA could contribute to chronic intestinal inflammation through stimulation of dsDNA responsive inflammasomes. Besides commensal bacteria, the gut mucosa is continuously exposed to a variety of pathogens[38]. Interestingly, several studies demonstrated inflammasome involvement in response to invading pathogens. For example, the NLRP3 inflammasome is essential for the clearance of infections with pathogenic bacteria such as *Staphylococcus aureus*, *Vibrio cholerae*, *Escherichia coli*, *Neisseria gonorrhoeae*, *Chlamydia pneumoniae* and *Citrobacter rodentium*[39], while AIM2 has been shown to be activated by *Lysteria monocytogenes* and *Francisella tularensis*[39]. Some of these bacteria can be found in the gut, as they are the cause of intestinal bacterial infections.

Both *AIM2* and *IFI16* expression can be induced by type I and/or type II interferons (IFN)[40], which are the first line of defense against viral pathogens[41]. Increased IFN-γ levels have been associated with CD[42], and the influence of the intestinal virome on IBD has recently been intensely investigated[43]. Foxman & Iwasaki suggested that highly prevalent viruses could be a partial causative factor of chronic diseases such as IBD[43]. Also, virus-induced intestinal inflammation has been linked to defective autophagy in a mouse model of colitis[44]. Paneth cell abnormalities such as secretion defects and morphological changes can be seen in biopsies from mice with hypoactive ATG16L1, and patients carrying the CD-associated *ATG16L1* allele[45]. More recently, it has been shown that a viral infection is needed for the CD-associated Paneth cell phenotype in mice. Interestingly, antibiotics could prevent this phenotype indicating that a bacterial component is also needed in this model[44]. The authors thus suggest a multi-hit model for the development of IBD where microbial, viral and genetic components coincide[44, 45]. It will be important/interesting to further explore the role of AIM2 and IFI16 for example by using knockout mice in the described IBD models. To our knowledge, only AIM2 knockout mice have been used to investigate the role of this protein in the response to bacterial and viral infections. Fernandes-Alnemri *et al.* showed that AIM2 knockout mice are more susceptible to infection by *F. tularensis*, while Rathinam and collaborators showed an altered IL-18 secretion in these mice after cytomegalovirus infection[46, 47].

Our findings could have important implications with regard to more personalized therapies, which seems to become the future of IBD treatment[48]. For example, patients carrying mutations in one of the components in the microbial response (e.g. autophagy, IFN-signaling, PYHIN-inflammasomes and their effectors) might have difficulties in tolerating commensals and clearing intracellular pathogens, which could contribute to the induction and chronic maintenance of intestinal inflammation, and thus IBD pathogenesis. Furthermore, patients that carry additional mutations in bacterial clearing genes might have an even worse prognosis. Inflammasomes, mainly NLRP3 have already been investigated in animal models of IBD. Depending on the study, investigators identified aggravating or beneficial effects of NLRP3 knockout on intestinal inflammation[49, 50]. Later, Bauer *et al.* proposed that these contradicting findings might be the consequence of the presence of distinct micro-organisms in different animal facilities[7]. It could for example be the presence of a microbial/viral component that has an additive effect on the chemically-induced intestinal inflammation in

NLRP3 knockout mice in this experimental setup. Different microbial and/or viral composition of the gut might also explain why the *NLRP3* association with IBD[12] could not be replicated[51].

With regard to current IBD treatments, in this study we show that patients with colonic mucosal healing after infliximab treatment have a significant drop in the mucosal expression of the PYHIN inflammasome genes, while this is not the case for patients who do not respond to anti-TNF therapy. Therefore, although preliminary, it is tempting to speculate about potential clinical implications. For example, increased *AIM2* and *IFI16*-expression in mucosal biopsies, and possibly also in blood immune cells, may hold potential as a biomarker for non-responsiveness to therapy. Also, the fact that infliximab non-responsive patients show a sustained high expression of PYHIN inflammasomes could be an indication that in these patients a stronger viral component is present. Another interesting finding was the persistent up-regulation of *IFI16* colonic gene expression in inactive IBD, and in IBD responders with mucosal healing. This may be one of the reasons why mucosal ulcers recur very early if patients do not receive maintenance therapy to control the intestinal inflammation.

Our data do not allow us to determine whether *IFI16* and *AIM2* upregulation is either a cause or consequence of intestinal inflammation. It should be mentioned however that, independent from the sequence of events, the expression or activation of the inflammasome pathway holds potential as a biological marker or therapeutic target.

In conclusion, our findings firstly suggest a new link between the pro-inflammatory PYHIN inflammasomes and IBD pathogenesis. Secondly, response to infliximab is associated with expression levels of PYHIN inflammasome components. Our study thus indicates that it is worthwhile to further investigate/validate (PYHIN-based) inflammasome signaling and its association with the microbial/viral gut environment as potential therapeutic target.

6. Supplementary material

6.1. Protocol details

6.1.1. Whole-genome gene expression analysis

Briefly, in the first cycle, double stranded cDNA was prepared with random hexamers tagged with a T7 promoter sequence, followed by the generation of cRNA using the GeneChip WT Synthesis and Amplification kit (Ambion WT expression kit). cRNA concentration after cleanup was measured with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). In the second cycle, sense oriented single-stranded DNA containing dUTP was generated and the concentration is measured after clean-up using the NanoDrop. The cRNA is hydrolyzed and the single-stranded DNA is fragmented using uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE1) (GeneChip WT terminal Labeling kit, Affymetrix). The quality of fragmentation (fragments should be between 40 and 70 nucleotides) is checked on the 2100 Bioanalyzer (Agilent). The fragmented DNA is labeled by terminal deoxynucleotidyl transferase (TDT) with the Affymetrix DNA Labeling reagent that is covalently linked to biotin (GeneChip WT terminal Labeling kit, Affymetrix). Labeled DNA was hybridized to the array during 17h at 45°C. The arrays were washed and stained in a fluidics station using the GeneChip hybridization, Wash end Stain kit (Affymetrix) and scanned using the Affymetrix 3000 GeneScanner. All image files were generated using AGCC. Quality assessment and outlier detection was performed before and after normalization using the Bioconductor package arrayQualityMetrics[52].

6.1.2. qRT-PCR analysis

After cDNA synthesis from total RNA (0.5 µg) using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany), multiplex real-time PCR was performed in a total reaction volume of 25 µl on a Rotor-Gene 3000 instrument (Corbett Research, Mortlake, Australia) using the QuantiTect Multiplex PCR NoROX Kit (Qiagen, Venlo, NL). All samples were amplified in duplicate reactions.

6.1.3. Immunohistochemistry

After drying, deparaffinization and rehydration, epitope retrieval was performed at low pH for AIM2, and at high pH for IFI16 and CASP1 (Dako PT Link machine, Dako Belgium NV, Heverlee, Belgium). Sections were then washed 3 times for 5 min (Envision Flex wash buffer, Dako) and

Envision Flex Peroxidase-Blocking Reagent (Dako) was applied for 10 min at room temperature. After a second wash step, sections were incubated with an anti-human AIM2 rabbit polyclonal antibody (Sigma-Aldrich, Bornem, Belgium; dilution 3.2 µg/ml), or with an anti-human IFI16 rabbit polyclonal antibody (Sigma-Aldrich, dilution 0.5 µg/ml), or with anti-human CASP1 mouse monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany; dilution 2 µg/ml), or with anti-human HMGB1 rabbit polyclonal antibody (Abcam, Cambridge, UK; dilution 1 µg/ml) for 30 min at room temperature. Following a third wash step, bound primary antibody was visualized by incubating the slides for 30 min with Envision Flex/HRP (Dako) and application of the Envision DAB+ Chromogen (Dako) for 10 min at room temperature. After rinsing, the slides were counterstained with haematoxylin, dehydrated, cleared and mounted. Negative controls (no application of primary antibody) were run together with the test samples.

Microscopical images were acquired with the Cell* software (Olympus Soft Imaging Solutions GmbH, Münster, Germany) using a Olympus SC30 camera (Olympus Corporation, Tokyo, Japan) mounted on a Olympus BX41 bright field microscope. Total magnification was 100x and the objective lens used is a UPlamSApo 10x/0.40na; ∞/0.17/FN26.5 from Olympus.

6.2. Supplementary tables

Supplementary table 1: Characteristics of patients and controls in cohort 1.
**: immunosupperessants = azathioprine/6-mercaptopurine and methotrexate, IQR: interquartile range, IFX: infliximab, NA: not applicable*

Characteristics	UC		CD	Controls
	Active	Inactive	Active	
	(n=74)	(n=23)	(n=8)	(n=11)
Male/Female (%)	43/31 (58.1/41.9)	12/11 (52.2/47.8)	2/6 (25/75)	5/6 (45.5/54.5)
Median (IQR)* age (years)	45.9 (33.19-54.97)	43.93 (29.6-57.2)	38.9 (35.1-45.3)	69.6 (64-74.9)
Median (IQR)* duration of disease (years)	5.46 (2.59-13.5)	8.65 (3.3-17.9)	2 (0,19-3.99)	NA
Extent of disease				
UC Left-sided colitis (%)	35 (47.3)	13 (56.5)	NA	NA
Pancolitis (%)	39 (52.7)	10 (43.5)	NA	NA
CD Ileocolon (%)	NA	NA	3 (37.5)	NA
Ileum (%)	NA	NA	0 (0)	NA
Colon (%)	NA	NA	5 (62.5)	NA
Concomitant medication at first IFX (%)				
5-Aminosalicylates	59 (79.7)	22 (95.7)	1 (12.5)	NA
Corticosteroids	31 (41.9)	2 (8.7)	2 (25)	NA
Azathioprine/6-Mercaptopurine	13 (17.6)	10 (43.5)	0 (0)	NA
Methotrexate	2 (2.7)	0 (0)	0 (0)	NA
Anti-TNF	0 (0)	11 (47.8)	0 (0)	NA
Active smoking (%)	9 (12.2)	3 (13)	3 (37.5)	0 (0)

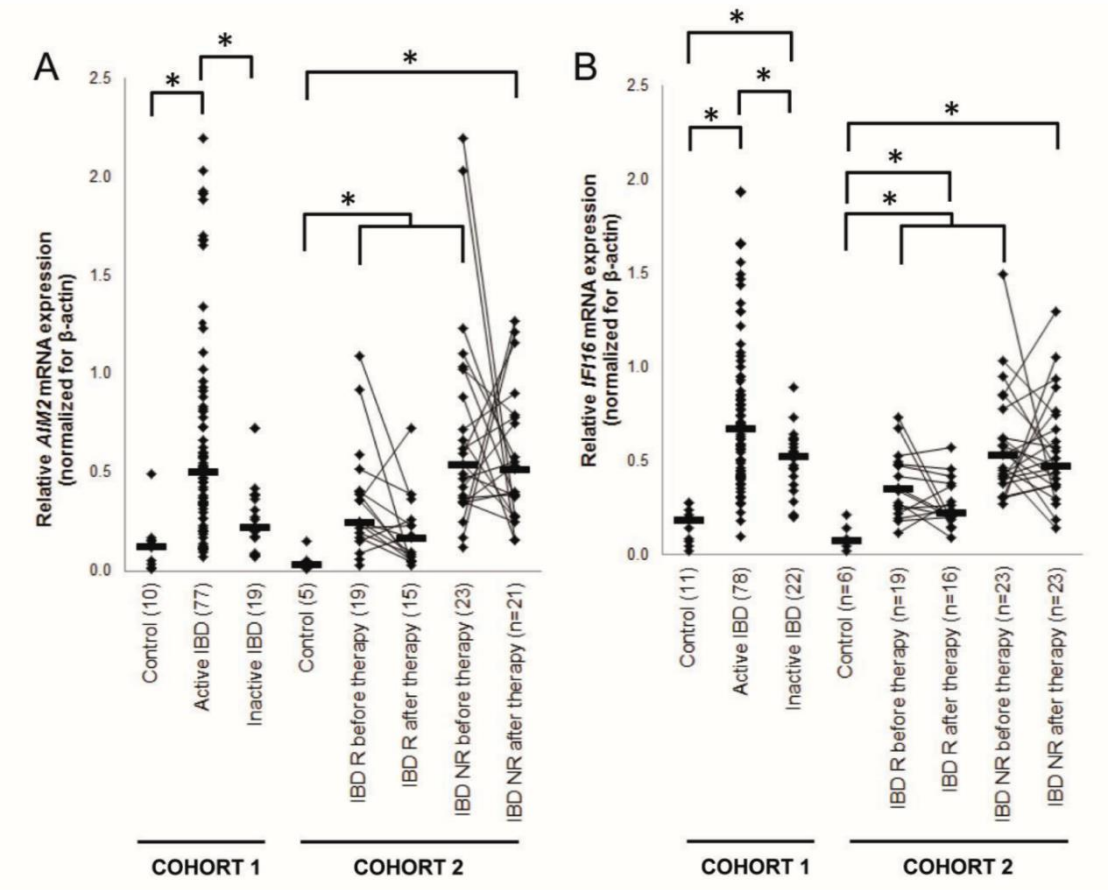
Supplementary table2: Primer and probe oligonucleotide sequences for qRT-PCR analyses of AIM2, IFI16 and β-actin.

Gene symbol		Sequence
AIM2	forward primer	5'-TCCTCATGTTAAGCCTGAACAG-3'
	reverse primer	5'-CTTTCAGTACCATAACTGGCAAAC-3'
	Probe	5'-TCTGATAGATTCCTGCTGGGCCACCATCTG-3'
IFI16	forward primer	5'-AGATAATACAGGGAAGATGGAAGT -3'
	reverse primer	5'-GTCTTGATGACCTTGATGTGACT-3'
	Probe	5'-TGGTGCATGGACGACTGACCACAATCAACT-3'
β-actin	forward primer	5'-CCCAGCACAATGAAGATCAAGATC-3'
	reverse primer	5'-CTGATCCACATCTGCTGGAAG-3'
	Probe	5'-CCTCCTGAGCGCAAGTACTCCGTGTG-3'

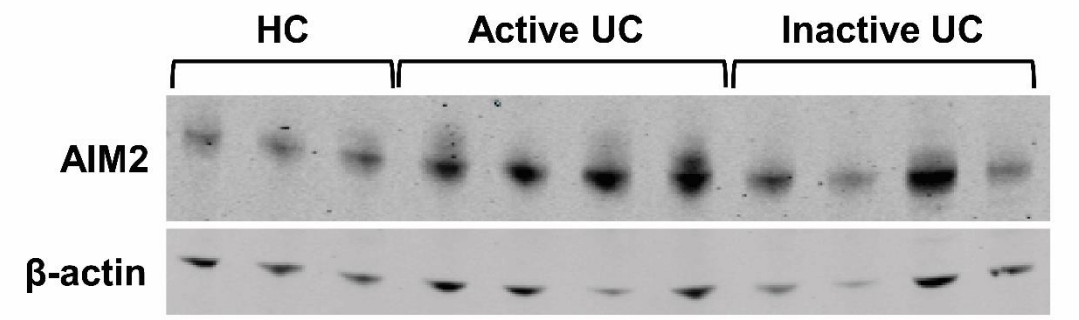
*Supplementary table 3: Statistical analyses (P-values) of the qRT-PCR data of AIM2 and IFI16 in colonic mucosa of controls and IBD patients. *significant P-value (P < 0.01), R: responders, NR: non-responders.*

	Comparison	*AIM2*	*IFI16*
		p-value	p-value
COHORT 1	active IBD vs control	<0.001*	<0.001*
	inactive IBD vs control colon	0,015	<0.001*
	inactive IBD vs active IBD	<0.001*	0.009*
COHORT 2	IBD before therapy vs control colons	0.001*	<0.001*
	IBD R after therapy vs control colons	0,021	0.005*
	IBD NR after therapy vs control colons	0.001*	<0.001*
	IBD R after therapy vs IBDc R before therapy	0,027	0,173
	IBD NR after therapy vs IBDc NR before therapy	0,357	0,738

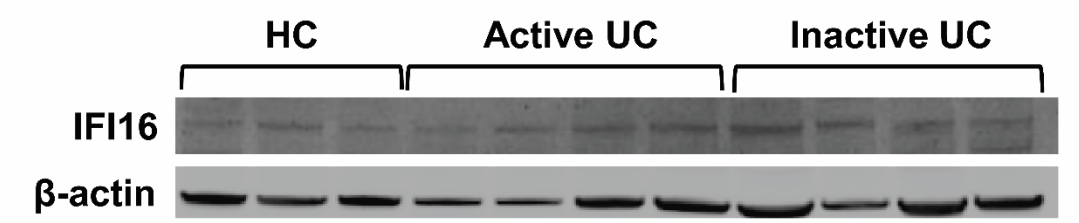
6.3. Supplementary figures



Supplementary figure 1: *qRT-PCR analysis of **A)** AIM2 and **B)** IFI16 in colonic mucosa from IBD patients and controls. A line between 2 points represents the change in expression before and after infliximab therapy for one patient. Individual expression levels (♦) and median (—) for each group are shown.*



Supplementary figure 2: *Western blot for detection and quantification of AIM2 in colonic whole biopsy lysates from healthy controls (HC;n=3) and patients with active (n=4) or inactive (n=4) UC.*



Supplementary figure 3: *Western blot for detection and quantification of IFI16 in colonic whole biopsy lysates from healthy controls (HC;n=3) and patients with active (n=4) or inactive (n=4) UC.*

7. References

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CHAPTER 6

MUCOSAL EXPRESSION OF AUTOPHAGY AND ER STRESS GENES IN INFLAMMATORY BOWEL DISEASES

CHAPTER 6: MUCOSAL EXPRESSION OF AUTOPHAGY AND ER STRESS GENES IN INFLAMMATORY BOWEL DISEASES

1. Introduction and aim

Endoplasmic reticulum stress and autophagy are two important processes that help to maintain intestinal homeostasis and have been identified as underlying pathophysiologic pathways in inflammatory bowel diseases (IBD). Besides being genetically associated to IBD, animal models and human histopathologic studies confirmed that (genetic) perturbations of these pathways lead to severe abnormalities in the intestinal mucosa and inflammation[1-9]. These pathways are now considered to be potential therapeutic targets in IBD[10-19]. A quantitative and qualitative assessment of these underlying pathophysiologic processes, might aid in therapeutic decision-making and prediction of disease course[20-22]. We investigate the link between ER stress and autophagy associated gene expression and disease activity in IBD or response to anti-TNF therapy. We used the same two cohorts that were used to study the inflammasome genes in Vanhove *et al.*[23] (**CHAPTER 5**).

2. Material and methods

This chapter is an addendum to CHAPTER 5, we refer to the material and methods section of that chapter for the methodological information on patients and biopsy specimens; RNA isolation; whole-genome gene expression analysis and ethical considerations. The selection and filtering of ER stress and autophagy genes, which is unique to this chapter, are described here.

2.1. Selection of ER stress and autophagy associated genes

We analyzed the mRNA expression of genes encoding proteins that are attributed to ER stress or autophagy according to the curated Reactome database (www.reactome.org) in endoscopic-derived colonic mucosal biopsies from patients in the two cohorts, with the use of Affymetrix whole genome gene expression microarrays.

We used the Reactome database to acquire a curated list of genes that are involved in ER stress or autophagy (86 and 69 genes, respectively, **Supplementary table 1 and 2**). After matching these genes to their respective probeset(s) on both microarray platforms, the same comparative analyses were performed as described in Vanhove *et al.*[23] and these results were used to filter and retain the strongest (top-10) significantly (FDR<0.05) upregulated and downregulated genes and their annotated probesets from each comparison. Furthermore, we filtered out genes that did not show consistent results in both cohorts in order to retain genes that were dysregulated in more than one cohort population. Genes showing strong fold changes between two patient groups in two independent cohorts, will be further discussed in this chapter.

3. Results

3.1. ER stress genes

We narrowed down the Reactome-based list of 86 ER stress genes to 23 by applying the described filtering steps, details of this filtering process can be found in supplement (5.1 and **supplementary table 1**). The refined lists of 23 ER stress genes (26 and 52 probesets in cohort 1 and 2, respectively) are listed in **Tables 1** and **2**. In cohort 1 (**Table1**), the genes with most pronounced statistically significant fold changes in patients with active colonic disease (*vs.* colonic tissue from healthy controls) were *KDELR3*, *XBP1* and *DNAJB9* (≥2-fold between active colonic disease and control colons) followed by *SSR1*, *HSP90B1*, *PDIA5* and *HYOU1* (≥1.7-fold) and *DNAJB11*, *HSPA5*, *SYVN1*, *FKBP14*, *SEC61A1*, *CALR*, *PDIA6* and *WFS1* (≥1.5-fold). The expression of all of these genes was decreased in patients with inactive disease and most of them normalized to the levels of healthy controls. However, *KDELR3*, *XBP1*, *SSR1*, *PDIA5*, *SYVN1*, *SEC61A1* were still increased in patients with inactive disease when compared to control colons.

Table 1: Fold changes of the gene probe sets (GeneChip® Human Gene 1.0 ST arrays) encoding the ER stress genes from the comparative analyses performed in cohort 1 for UC, CD and IBD.

gene probe set ID	gene Symbol	active versus control colons (n=11)			inactive versus control colons (n=11)			active (A) versus inactive (IA)		
		UC (n=74)	CD (n=8)	IBD (n=82)	UC (n=23)	CD (n=0)	IBD (n=23)	UC (n_A=74, n_{IA}=23)	CD (n_A=8, n_{IA}=0)	IBD (n_A=82, n_{IA}=23)
8004271	*ACADVL*	**0,72****	0,85	**0,73****	**0,71***	NA	**0,71***	1,01	**NA**	1,02
8002041	*ATP6V0D1*	**0,72****	0,83	**0,73****	**0,77****	NA	**0,77****	0,95	**NA**	0,96
8026106	*CALR*	1,51**	1,37**	1,50**	1,03	NA	1,03	1,47**	**NA**	1,45**
7964579	*CTDSP2*	**0,71****	**0,82***	**0,72****	**0,81***	NA	**0,81***	**0,88***	**NA**	**0,89***
8084634	*DNAJB11*	**1,66****	**1,39***	**1,63****	1,09	NA	1,09	**1,52****	**NA**	**1,49****
8135480	*DNAJB9*	**2,06****	**1,50***	**1,99****	1,09	NA	1,09	**1,88****	**NA**	**1,83****
7969651	*DNAJC3*	1,41**	1,16	1,38**	0,87	NA	0,87	**1,62****	**NA**	**1,59****
8145624	*EXTL3*	1,39**	1,24*	1,37**	**1,20***	NA	**1,20***	1,16**	**NA**	1,14**
8138834	*FKBP14*	1,56**	1,49	1,56**	1,03	NA	1,03	**1,52****	**NA**	**1,52****
7995895	*HERPUD1*	1,33*	1,06	1,30*	0,86	NA	0,86	**1,55****	**NA**	**1,52****
7958130	*HSP90B1*	**1,73****	**1,57****	**1,72****	1,07	NA	1,07	**1,62****	**NA**	**1,61****
8164165	*HSPA5*	**1,62****	1,34*	1,59**	1,09	NA	1,09	1,49**	**NA**	1,46**
7952145	*HYOU1*	**1,71****	**1,45***	**1,68****	1,09	NA	1,09	**1,56****	**NA**	**1,53****
8073015	*KDELR3*	**2,86****	**2,06****	**2,77****	**1,78***	NA	**1,78***	**1,61****	**NA**	**1,56****
8119648	*KLHDC3*	**0,78****	**0,77***	**0,78****	1,03	NA	1,03	**0,76****	**NA**	**0,76****
7900468	*NFYC*	**0,75****	**0,82***	**0,75****	**0,86***	NA	**0,86***	**0,87****	**NA**	**0,88****
7915345	*NFYC*	**1,02**	**1,02**	**1,02**	**1,04**	NA	**1,04**	**0,98**	**NA**	**0,98**
8082133	*PDIA5*	**1,74****	**1,51***	**1,72****	**1,55****	NA	**1,55****	1,12*	**NA**	1,11*
8040249	*PDIA6*	**0,78****	**0,76***	**0,78****	1,02	NA	1,02	**0,77****	**NA**	**0,77****
8050278	*PDIA6*	**1,50****	**1,29**	**1,48****	1	NA	1	**1,51****	**NA**	**1,49****
8082408	*SEC61A1*	1,53**	1,32*	1,51**	**1,16***	NA	**1,16***	1,33**	**NA**	1,31**
7952557	*SRPR*	1,46**	1,24	1,44**	1,05	NA	1,05	1,40**	**NA**	1,37**
8123767	*SSR1*	**1,86****	**1,66***	**1,84****	**1,25***	NA	**1,25***	1,49**	**NA**	1,47**
7949383	*SYVN1*	1,61**	**1,41****	**1,59****	**1,20***	NA	**1,20***	1,34**	**NA**	1,32**
8093906	*WFS1*	1,50**	1,23	1,47**	**1,20***	NA	**1,20***	1,25**	**NA**	1,23**
8075182	*XBP1*	**2,64****	**2,22****	**2,60****	**1,48****	NA	**1,48****	**1,79****	**NA**	**1,76****

**: FDR<0.05, **: FDR<0.001, NA: not applicable. Probesets belonging to genes that were in the top-10 up/downregulated genes in a given comparison are indicated in bold.*

Most findings in cohort 1 were confirmed in cohort 2 (**Table 2**), *KDELR3*, *XBP1*, *DNAJB9*, *HSP90B1* and *CALR* expression was significantly increased (≥2-fold) in patients with active colonic disease (before infliximab) when compared to control colon. Other highly upregulated genes from cohort 1 such as *SSR1*, *PDIA5*, *PDIA6*, *WFS1*, *SRPR*, *HSPA5* and *HYOU1* were >1.5-fold upregulated in patients with active colonic disease when compared to control colons. Most genes also normalized after successful IFX therapy (R after infliximab) except for *KDELR3* and *XBP1* which remained significantly upregulated. In non-responders to IFX, all the aforementioned genes remained significantly increased after anti-TNF therapy.

Finally, we found that, before therapy, a specific subset of ER stress genes is significantly increased in patients who will not respond to IFX when compared to patients who will respond: *DNAJB9, XBP1, HSP90B1, FKBP14, SRPR, WFS1* and *HERPUD1*. Similar predictive transcriptomic profiles have already been discovered in this cohort[24, 25]. The current findings suggest that this ER stress-specific signature might be of additive value to the previously-identified signature.

Table 2: Fold changes of the probe sets (Human Genome U133 Plus 2.0 arrays) encoding the ER stress genes from the performed comparative analyses in cohort 2 for UC, CD and IBD.

probe set ID	gene symbol	before infliximab versus control colons (n=6)			R after infliximab versus control colons (n=6)			NR after infliximab versus control colons (n=6)			R after infliximab versus R before infliximab			NR after infliximab versus NR before infliximab			NR before infliximab versus R before infliximab		
		UC (n=24)	CD (n=19)	IBD (n=43)	UC (n=8)	CD (n=11)	IBD (n=19)	UC (n=16)	CD (n=7)	IBD (n=23)	UC (n=8)	CD (n=11)	IBD (n=19)	UC (n=16)	CD (n=7)	IBD (n=23)	UC (n_{NR}=16; n_R=8)	CD (n_{NR}=7; n_R=12)	IBD (n_{NR}=23; n_R=20)
200710_at	*ACADVL*	**0,60****	**0,58****	**0,59****	0,67	**0,66***	**0,66***	**0,62****	**0,58***	**0,60****	1	1,14	1,08	1,08	1,01	1,06	0,86	0,97	0,93
212041_at	*ATP6V0D1*	**0,71***	**0,72***	**0,72***	0,68	0,72	**0,70***	**0,63****	**0,71***	**0,66****	0,80*	0,97	0,89	0,97	1,01	0,98	0,77	0,97	0,86
200935_at	*CALR*	**2,19****	**2,03****	**2,11****	1,2	1,44	1,33	**1,88***	1,79*	1,85*	**0,53***	0,76	**0,65***	0,88	0,83	0,86	0,94	1,1	1,03
214315_x_a	*CALR*	**1,34**	**1,53**	**1,43**	1,27	1,49	1,4	**1,43**	1,43*	1,43	**0,97**	1,11	**1,05**	1,05	0,92	1,01	1,04	1,03	0,99
212953_x_a	*CALR*	**1,33***	**1,41**	**1,36***	1,15	1,31	1,24	**1,29**	1,40*	1,32*	**0,8**	0,98	**0,9**	1,01	0,99	1	0,89	1,01	0,93
212952_at	*CALR*	**1,2**	**1,19**	1,2	1,03	1,08	1,06	**1,41***	1	1,27	**0,89**	0,85	**0,87**	1,15	0,88	1,06	1,05	0,93	1
214316_x_a	*CALR*	**1,14**	**1,17**	1,15	0,77	0,99	0,89	**1,33**	1,03	1,23	**0,63**	0,81	**0,73***	1,2	0,89	1,09	0,91	0,99	0,94
203445_s_a	*CTDSP2*	**0,65****	**0,71****	**0,68****	0,72	0,73	**0,73***	**0,63****	**0,61***	**0,63****	1,07	0,99	1,02	0,99	0,92	0,97	0,95	0,9	0,91
208735_s_a	*CTDSP2*	**0,76***	**0,81**	**0,78***	0,75	0,78	**0,77**	**0,71***	**0,73***	**0,71***	0,97	0,92	0,94	0,94	0,95	0,94	0,97	0,91	0,93
223054_at	*DNAJB11*	1,81**	1,72*	1,77**	1,07	1,24	1,17	1,70**	1,79**	1,73**	**0,63***	0,74	**0,69***	0,91	0,96	0,92	1,11	1,13	1,13
202843_at	*DNAJB9*	**3,62****	**3,35****	**3,50****	1,51	1,93	1,74	**3,01****	**3,92****	**3,26****	**0,57**	**0,66**	**0,62***	0,71	0,9	0,76	1,61	1,52	**1,54***
202842_s_a	*DNAJB9*	**3,07****	**2,61****	**2,86****	1,28	1,46	1,38	**2,38****	**2,95****	**2,54****	**0,53***	**0,65***	**0,60****	0,69	0,87	0,74	1,44	1,5	**1,49***
1554462_a_	*DNAJB9*	**2,52****	**2,12***	**2,33****	1,22	1,34	1,29	**2,06***	**2,26***	**2,12****	**0,59***	**0,76**	**0,69***	0,74	0,75	0,74	1,35	1,74	**1,54***
208499_s_a	*DNAJC3*	**2,39****	**1,90***	**2,16****	1,22	1,5	1,38	**1,97***	**1,97***	**1,97****	**0,55****	0,83	**0,70***	0,79	0,87	0,81	1,13	1,33	1,28
209202_s_a	*EXTL3*	0,87	**0,72****	0,80*	0,9	0,85	0,87	0,89	**0,76***	0,85	1,01	1,2	1,12	1,04	1,01	1,03	0,97	1,07	1,07
211051_s_a	*EXTL3*	1,20*	**1,07**	1,14	0,99	1,09	1,05	1,12	1	1,08	0,89	1,04	0,97	0,9	0,93	0,91	1,11	1,02	1,1
219390_at	*FKBP14*	1,74*	1,68*	1,71*	1,12	1,25	1,19	1,75*	**2,20***	**1,88***	0,87	0,87	0,87	0,87	1,06	0,92	1,57	1,39	**1,45***
235311_at	*FKBP14*	1,15	1,07	1,11	0,96	0,98	0,97	1,08	**1,08**	**1,08**	0,93	0,96	0,95	0,89	0,97	0,91	1,18	1,07	1,14
217168_s_a	*HERPUD1*	1,42*	1,24	1,34	1,02	0,95	0,98	1,21	1,44	1,28	0,86	0,87	0,87	0,78	0,97	0,83	1,31	1,33	**1,34***
216450_x_a	*HSP90B1*	**3,71****	**3,30***	**3,52****	1,84	2,06	1,96	**3,14***	**3,07***	**3,12****	**0,56***	0,56	**0,56****	0,8	0,94	0,84	1,19	0,99	1,13
200598_s_a	*HSP90B1*	**2,64****	**2,31****	**2,49****	1,27	1,54	1,42	**2,30****	**2,12****	**2,25****	**0,55***	0,7	**0,64****	0,81	0,8	0,81	1,23	1,26	**1,27***
216449_x_a	*HSP90B1*	**2,18****	**1,95***	**2,07****	1,21	1,41	1,32	**2,05****	**1,84***	**1,99****	**0,59***	0,75	**0,67****	0,92	0,83	0,89	1,08	1,23	1,17
200599_s_a	*HSP90B1*	**1,95****	**1,74***	**1,85****	1,14	1,35	1,26	**1,84****	**1,80***	**1,82****	**0,63***	0,84	**0,74***	0,91	0,89	0,9	1,11	1,27	**1,20***
211936_at	*HSPA5*	1,98**	**1,79****	**1,89****	1,25	1,43	1,35	**1,94****	**1,85****	**1,91****	**0,67***	0,85	0,77*	0,95	0,94	0,95	1,09	1,16	1,15
230031_at	*HSPA5*	1,63**	**1,58****	**1,61****	1,23	1,49	1,38	**1,79****	**1,79***	**1,79****	**0,8**	0,98	0,9	1,06	1,02	1,05	1,1	1,18	1,13
200825_s_a	*HYOU1*	1,76**	1,62**	1,70**	1,02	1,2	1,12	1,66**	1,55*	1,62**	**0,58***	0,75	**0,67***	0,94	0,93	0,94	1,02	1,03	1,05

**: FDR<0.05, **: FDR<0.001, R: responders, NR: non-responders. Probesets belonging to genes that were in the top-10 up/downregulated genes in a given comparison are indicated in bold.*

Table 2-continued: Fold changes of the probe sets (Human Genome U133 Plus 2.0 arrays) encoding the ER stress genes from the performed comparative analyses in cohort 2 for UC, CD and IBD.

probe set ID	gene symbol	before infliximab versus control colons (n=6)			R after infliximab versus control colons (n=6)			NR after infliximab versus control colons (n=6)			R after infliximab versus R before infliximab			NR after infliximab versus NR before infliximab			NR before infliximab versus R before infliximab		
		UC (n=24)	CD (n=19)	IBD (n=43)	UC (n=8)	CD (n=11)	IBD (n=19)	UC (n=16)	CD (n=7)	IBD (n=23)	UC (n=8)	CD (n=11)	IBD (n=19)	UC (n=16)	CD (n=7)	IBD (n=23)	UC (n_{NR}=16; n_R=8)	CD (n_{NR}=7; n_R=12)	IBD (n_{NR}=23; n_R=20)
204017_at	*KDELR3*	**4,64****	**3,94****	**4,32****	2,37	**2,63***	**2,52***	**4,21****	**3,98***	**4,14****	**0,50***	0,71	**0,61***	0,92	0,88	0,91	0,96	1,25	1,13
207265_s_a	*KDELR3*	**4,07****	**3,50****	**3,81****	1,95	**2,17**	**2,07***	**3,53****	**3,54***	**3,53****	**0,50***	0,68	**0,60***	0,85	0,85	0,85	1,08	1,3	1,21
207264_at	*KDELR3*	**2,44****	**2,29****	**2,37****	1,45	**1,6**	**1,53**	**2,00***	**2,41***	**2,12***	**0,57***	0,77	**0,68***	0,84	0,87	0,85	0,94	1,36	1,12
208784_s_a	*KLHDC3*	**0,77***	0,78*	**0,78***	1	0,85	0,91	**0,78***	**0,75***	**0,77***	**1,25***	1,1	**1,16***	1,03	0,98	1,02	0,95	0,97	0,96
214383_x_a	*KLHDC3*	**0,83***	0,83*	**0,83***	1,01	0,95	0,97	**0,84**	0,8	**0,83**	**1,23**	1,13	**1,17**	1,01	0,99	1,01	1,02	0,96	0,99
211251_x_a	*NFYC*	**0,73***	**0,71***	**0,72***	0,72	0,75	0,74	**0,68***	**0,72***	**0,69***	1,05	1,02	1,03	0,92	0,99	0,94	1,09	1,02	1,06
202216_x_a	*NFYC*	**0,76***	**0,74***	**0,75***	0,74	0,8	0,78	**0,72**	**0,76***	**0,73***	1,08	1,06	1,07	0,9	1,02	0,94	1,15	1,01	1,09
202215_s_a	*NFYC*	**0,75***	**0,83**	**0,78***	0,87	0,93	0,9	**0,72***	**0,81**	**0,75***	1,16	1,1	1,12	0,96	1,06	0,99	1	0,88	0,92
211797_s_a	*NFYC*	**0,83**	**0,78**	**0,81**	0,78	0,77	0,77	**0,73**	**0,76**	0,74	0,96	0,96	0,96	0,87	0,92	0,89	1,03	1,08	1,07
238231_at	*NFYC*	**0,96**	**1**	**0,98**	1,05	1	1,02	**0,99**	**0,96**	**0,98**	1,06	1,02	1,04	1,04	0,96	1,02	0,96	1,01	0,97
203857_s_a	*PDIA5*	**2,00****	**1,82****	**1,92****	1,5	1,46	1,48	**1,89****	**1,81***	**1,87****	0,81	0,84	0,83	0,91	0,94	0,92	1,11	1,09	1,13
216640_s_a	*PDIA6*	**1,86****	1,60*	1,74**	1,05	1,15	1,11	1,64*	1,57*	**1,62****	**0,61***	0,76	**0,69***	0,85	0,85	0,85	1,12	1,24	1,21
208639_x_a	*PDIA6*	**1,80****	1,57*	1,69**	1,07	1,2	1,15	1,61*	1,54*	1,59**	**0,63***	0,81	**0,73***	0,87	0,88	0,88	1,08	1,18	1,16
207668_x_a	*PDIA6*	**1,76****	1,60*	1,69**	1,04	1,21	1,14	1,62*	1,57*	1,61**	**0,62***	0,79	**0,71***	0,91	0,9	0,9	1,06	1,14	1,12
208638_at	*PDIA6*	**1,50****	1,44*	1,48**	1,06	1,2	1,14	1,51*	1,39*	1,47**	**0,75***	0,88	**0,83**	0,98	0,85	0,94	1,1	1,22	1,15
200917_s_a	*SRPR*	1,67**	1,39	1,54*	1,05	1,04	1,04	1,37	1,43	1,39*	0,75*	0,79	0,77*	0,75	0,91	0,8	1,3	1,22	**1,31***
200918_s_a	*SRPR*	1,64**	1,41*	1,54**	1,08	1,11	1,1	1,49*	1,55*	1,50*	0,72*	0,84	0,79*	0,87	0,97	0,9	1,14	1,2	**1,21***
226712_at	*SSR1*	**2,13****	**1,78****	**1,96****	1,31	1,68	1,51	**1,95****	**1,83***	**1,91****	**0,72***	0,92	**0,83**	0,85	1,1	0,92	1,25	0,91	1,13
200889_s_a	*SSR1*	**2,04****	**1,71***	**1,89***	1,01	1,14	1,08	**1,71***	**1,66***	**1,69***	**0,53***	0,73	**0,64***	0,8	0,78	0,79	1,13	1,42	1,29
200890_s_a	*SSR1*	**1,77***	**1,5**	**1,65***	0,88	1,06	0,98	**1,55***	**1,44***	**1,52***	**0,54***	0,75	**0,65***	0,84	0,83	0,83	1,14	1,27	1,24
200891_s_a	*SSR1*	**1,57****	**1,56****	**1,57****	1,1	1,31	1,22	**1,60***	**1,51***	**1,57****	**0,73***	0,85	**0,80***	1	0,92	0,97	1,07	1,09	1,07
225435_at	*SSR1*	**1,53***	**1,23**	**1,39***	1,14	1,22	1,18	**1,53***	**1,2**	**1,42**	**0,83**	0,98	**0,92**	0,95	1	0,96	1,18	0,97	1,14
225090_at	*SYVN1*	**1,91****	1,68**	1,80**	1,22	1,33	1,28	**1,84****	1,72*	1,81**	0,72*	0,86	0,80*	0,91	0,91	0,91	1,19	1,2	1,22
244522_at	*SYVN1*	**0,85**	0,83	0,84*	0,9	0,87	0,88	**0,87**	0,88	0,87	1,02	1,01	1,01	1,03	1,08	1,05	0,95	0,98	0,98
202908_at	*WFS1*	1,65**	1,37*	1,52*	1,03	1,09	1,07	1,47*	1,68*	1,53*	0,68*	0,89	0,80*	0,85	1,04	0,91	1,14	1,3	**1,25***
200670_at	*XBP1*	**2,97****	**2,61****	**2,81****	1,8	1,83	**1,82***	**2,81****	**3,09***	**2,89****	0,78	0,77	0,77*	0,84	1,04	0,89	1,45	1,22	**1,36***

**: FDR<0.05, **: FDR<0.001, R: responders, NR: non-responders. Probesets belonging to genes that were in the top-10 up/downregulated genes in a given comparison are indicated in bold.*

3.2. Autophagy genes

We also narrowed down the list of 69 autophagy genes to 14 by applying the described filtering steps, details of this filtering process can be found in supplement (5.2 and **supplementary table 2**). The refined lists of 14 autophagy genes (15 and 33 probesets in cohort 1 and 2, respectively) are listed in **Table 3** and **4**.

Table 3: Fold changes of the gene probe sets (GeneChip® Human Gene 1.0 ST arrays) encoding the autophagy genes from the comparative analyses performed in cohort 1 for UC, CD and IBD.

gene probe set ID	gene Symbol	active versus control colons (n=11)			inactive versus control colons (n=11)			active (A) versus inactive (IA)		
		UC (n=74)	CD (n=8)	IBD (n=82)	UC (n=23)	CD (n=0)	IBD (n=23)	UC (n_A=74, n_{IA}=23)	CD (n_A=8, n_{IA}=0)	IBD (n_A=82, n_{IA}=23)
7979328	*ATG14*	**0,68****	**0,71****	**0,68****	**0,71****	NA	**0,71****	0,95	NA	0,95
8169272	*ATG4A*	1,04	0,93	1,03	**0,72***	NA	**0,72***	**1,45****	NA	**1,45****
8025659	*ATG4D*	**0,68****	0,83	**0,70****	0,93	NA	0,93	**0,73****	NA	**0,73****
8077858	*ATG7*	**1,21***	1,16	**1,20***	1,02	NA	1,02	**1,19****	NA	**1,19****
7955606	*C12orf44*	**1,18****	1,09	**1,17****	**1,19***	NA	**1,19***	0,99	NA	0,99
8039796	*CHMP2A*	**0,74****	**0,77***	**0,74****	**0,86***	NA	**0,86***	0,86**	NA	0,86**
8053562	*CHMP3*	0,78**	**0,82***	0,78**	0,97	NA	0,97	**0,80****	NA	**0,80****
8061958	*CHMP4B*	**0,73****	**0,78***	**0,73****	**0,86***	NA	**0,86***	**0,84****	NA	**0,84****
7954810	*LRRK2*	**2,86****	**2,70***	**2,85****	1,08	NA	1,08	**2,64****	NA	**2,64****
8062023	*MAP1LC3A*	**0,71****	0,87	**0,72****	1,02	NA	1,02	**0,70****	NA	**0,70****
8072302	*MTMR3*	**0,71****	**0,75****	**0,71****	**0,83***	NA	**0,83***	0,86**	NA	0,86**
8072279	*MTMR3*	**0,81****	**0,85***	**0,82****	**0,94**	NA	**0,94**	0,87**	NA	0,87**
7919305	*PRKAB2*	**0,61****	**0,66****	**0,61****	**0,74***	NA	**0,74***	**0,82****	NA	**0,82****
8150757	*RB1CC1*	0,80**	**0,79***	0,80**	**0,74****	NA	**0,74****	1,08	NA	1,08
8128123	*RRAGD*	1,28	1,17	1,27	0,93	NA	0,93	**1,38****	NA	**1,38****

**: FDR<0.05, **: FDR<0.001, NA: not applicable. Probesets belonging to genes that were in the top-10 up/downregulated genes in a given comparison are indicated in bold.*

Table 4: Fold changes of the probe sets (Human Genome U133 Plus 2.0 arrays) encoding the ER stress genes from the performed comparative analyses in cohort 2 for UC, CD and IBD.

probe set ID	gene symbol	before infliximab versus control colons (n=6)			R after infliximab versus control colons (n=6)			NR after infliximab versus control colons (n=6)			R after infliximab versus R before infliximab			NR after infliximab versus NR before infliximab			NR before infliximab versus R before infliximab		
		UC (n=24)	CD (n=19)	IBD (n=43)	UC (n=8)	CD (n=11)	IBD (n=19)	UC (n=16)	CD (n=7)	IBD (n=23)	UC (n=8)	CD (n=11)	IBD (n=19)	UC (n=16)	CD (n=7)	IBD (n=23)	UC (n_{NR}=16; n_R=8)	CD (n_{NR}=7; n_R=12)	IBD (n_{NR}=23; n_R=20)
204568_at	*KIAA0831*	**0,65****	**0,70***	**0,67****	0,62	**0,68***	0,66	**0,65***	**0,64***	**0,65****	0,98	1,01	1	0,99	0,91	0,96	0,96	0,99	1
213115_at	*ATG4A*	1,18	1,15	1,17	0,73	1,01	0,88	1,06	1,09	1,07	**0,64***	0,87	**0,76***	0,88	0,92	0,89	0,95	0,96	0,95
226871_s_at	*ATG4D*	**0,61****	**0,68***	**0,64***	0,71	0,83	0,78	**0,59****	0,65	**0,61****	1,06	1,13	1,1	1,02	1,12	1,05	1,16	1,3	1,24
218673_s_at	*ATG7*	**1,28***	**1,29***	**1,29***	0,92	1,09	1,02	1,17	**1,23***	**1,19***	**0,75***	0,88	**0,82***	0,9	0,91	0,9	0,94	0,92	0,94
237119_at	*ATG7*	**1,07**	**0,98**	**1,03**	1,02	1,08	1,05	1,07	**1,18**	**1,1**	**1,01**	1,1	**1,06**	0,98	1,18	1,03	0,93	0,97	0,93
222709_at	*ATG7*	**1,04**	**1,09**	**1,06**	0,95	1,05	1	0,99	**1,14**	**1,03**	**0,99**	0,97	**0,97**	0,92	1,05	0,95	0,89	1,02	0,96
224025_s_at	*ATG7*	**1,03**	**0,99**	**1,02**	0,86	0,95	0,91	0,96	**1,04**	**0,98**	**0,83**	0,96	**0,91**	0,92	1	0,94	0,99	0,94	0,96
1569827_at	*ATG7*	**1,02**	**1,06**	**1,04**	0,96	1,06	1,02	1,05	**1,03**	**1,04**	**0,88**	0,99	**0,94**	1,07	0,99	1,05	1,12	1,04	1,09
1556240_at	*ATG7*	**0,84***	**0,80***	**0,82***	0,85	0,83	0,84	0,81*	**0,85**	**0,82***	**0,97**	1,03	**1**	0,99	1,11	1,03	1,07	1,08	1,05
218214_at	*C12orf44*	**1,38****	1,19	**1,29***	1,19	1,15	1,17	**1,36****	**1,35***	**1,36****	0,89	1,04	0,97	0,98	1,04	0,99	0,96	0,87	0,89
202121_s_at	*CHMP2A*	0,71*	0,74*	0,72*	0,74	0,78	0,76	0,68*	0,70*	**0,69***	0,95	1,05	1,01	1,01	0,95	0,99	1,14	0,99	1,08
217837_s_at	*VPS24*	**0,65****	0,72**	**0,68****	0,84	0,85	0,85	0,71*	**0,66***	0,69**	**1,18**	1,15	**1,16***	1,15	0,95	1,09	1,15	1,06	1,13
222437_s_at	*VPS24*	**0,73***	0,82	**0,77***	0,97	0,95	0,96	0,80*	**0,75**	0,79*	**1,26***	1,1	**1,16***	1,12	1,03	1,1	1,08	1,21	1,16
222436_s_at	*VPS24*	**0,77***	0,81*	**0,79***	0,85	0,84	0,84	0,77*	**0,74***	0,76*	**1,01**	1,02	**1,02**	1,05	0,94	1,01	1,14	1,03	1,09
225498_at	*CHMP4B*	**0,45****	**0,52****	**0,48****	0,67	**0,67***	**0,67***	**0,49****	**0,48****	**0,49****	**1,34***	1,22	**1,27***	1,15	1,03	1,12	1,17	1,17	**1,20***
225119_at	*CHMP4B*	**0,57***	**0,46***	**0,52***	0,53	**0,53**	**0,53**	**0,52***	**0,47***	**0,50***	**0,86**	1,13	**1,01**	0,94	0,92	0,94	1,11	0,85	**0,93**
229584_at	*LRRK2*	**2,53***	**2,42***	**2,48***	1,08	1,09	1,09	**1,97***	**4,71***	**2,57***	**0,69***	**0,66***	**0,67****	0,62	1,05	0,73	0,49	**0,38***	**0,46****
232011_s_at	*MAP1LC3A*	**0,53****	**0,54****	**0,53****	0,68	**0,65***	**0,66***	**0,53****	**0,55****	**0,53****	1,34	1,24	**1,28***	0,99	0,97	0,98	0,95	0,92	0,95
227219_x_at	*MAP1LC3A*	**0,63****	**0,65****	**0,64****	0,77	**0,77**	**0,77**	**0,61****	**0,60***	**0,60****	1,16	1,2	**1,18***	1	0,89	0,96	1,09	0,95	1,04
224378_x_at	*MAP1LC3A*	**0,68****	**0,69****	**0,69****	0,83	**0,79**	**0,81**	**0,67****	0,70*	**0,68****	1,22	1,13	**1,17**	0,98	1,04	1	1	1,05	1,02
202198_s_at	*MTMR3*	0,71*	**0,70***	0,71*	0,74	0,77	0,76	**0,65***	**0,65***	**0,65***	**1,03**	1,11	**1,08**	0,92	0,86	0,9	1,01	0,9	0,96
211507_s_at	*MTMR3*	0,76*	**0,71***	0,74*	0,79	0,78	0,79	**0,69***	**0,69**	**0,69***	**1,01**	1,08	**1,05**	0,93	0,96	0,94	1,06	0,97	1
226956_at	*MTMR3*	0,69*	**0,74***	0,71**	0,88	0,86	0,87	**0,71****	**0,71***	**0,71****	**1,25***	1,14	**1,19***	1,04	0,95	1,01	1,03	1	1,04
240917_at	*MTMR3*	0,84*	**0,85**	0,84*	0,95	0,93	0,94	**0,87**	**0,75***	**0,83**	**1,04**	1,06	**1,05**	1,09	0,95	1,04	1,14	1,13	1,13
202197_at	*MTMR3*	0,84*	**0,88**	0,86*	0,78	0,82	0,8	**0,83**	**0,82**	**0,83***	**0,97**	0,94	**0,96**	0,96	0,94	0,95	0,93	1,01	0,98
240271_at	*MTMR3*	1,17	**1,16**	1,17	1,38	1,2	1,27	**1,37**	**1,35**	**1,36**	**1,18**	1,14	**1,15**	1,17	1,09	1,14	1	0,91	0,96
225278_at	*PRKAB2*	**0,57****	**0,63***	**0,59****	0,67	0,65	0,66	**0,55****	**0,56***	**0,55****	1,14	1,15	1,15	0,98	0,77	0,91	1,06	0,81	0,97
1558027_s_at	*PRKAB2*	**0,66***	0,72*	**0,69***	0,7	0,79	0,75	**0,71***	**0,65***	**0,69***	1,04	1,18	1,12	1,08	0,84	1	1,02	0,9	0,99
214474_at	*PRKAB2*	**0,88**	**0,88**	**0,88**	0,83	0,91	0,88	**0,78**	**0,76**	**0,78***	0,92	1,02	0,98	0,9	0,85	0,89	1,04	0,97	1,01
202033_s_at	*RB1CC1*	0,73*	0,72*	0,73*	0,67	0,73	0,7	**0,67***	**0,64***	**0,66***	0,99	1,05	1,03	0,88	0,82	0,86	0,89	0,89	0,9
202034_x_at	*RB1CC1*	0,77*	0,79	0,78	0,73	0,8	0,77	**0,79**	**0,86**	**0,81**	1,05	1,12	1,09	0,97	0,94	0,96	0,86	0,8	0,85
221524_s_at	*RRAGD*	**1,61***	**1,60***	**1,61***	1,01	0,99	1	1,35	1,59	1,42	**0,76***	0,69	**0,72***	0,76	0,84	0,78	0,75	0,77	**0,78***
221523_s_at	*RRAGD*	1,26	**1,29**	**1,28**	0,91	0,93	0,92	1,17	1,61	1,29	**0,92**	0,81	**0,85**	0,82	1,07	0,89	0,7	0,79	**0,76***

**: FDR<0.05, **: FDR<0.001, R: responders, NR: non-responders. Probesets belonging to genes that were in the top-10 up/downregulated genes in a given comparison are indicated in bold.*

In cohort 1 (**Table 3**) only *LRRK2* was significantly upregulated (> 2-fold) in patients with active disease when compared to control individuals and this was confirmed in cohort 2 (**Table 4**). All other autophagy genes with pronounced fold changes such as *PRKAB2*, *ATG14*/*KIAA0831*, *ATG4D*, *MTMR3*, *MAP1LC3A*, *CHMP4B* and *CHMP2A* were downregulated in patients with active disease in both cohorts. From these 7 genes, *PRKAB2*, *ATG14*/*KIAA0831*, *MTMR3*, *CHMP4B* and *CHMP2A* remain downregulated in patients with inactive disease (cohort 1) but this was only confirmed for *MAP1LC3A* and *CHMP4B* in the second cohort (after successful anti-TNF therapy).

Interestingly, *LRRK2* was differentially expressed when comparing IFX-responders and non-responders before the initiation of IFX therapy with a >2-fold downregulation in the latter group. This difference was only significant in CD patients or when grouping CD and UC patients together (FDR < 0.05 and < 0.001, respectively)

4. Discussion

Given the well-evidenced involvement of ER stress and autophagy in IBD, we analyzed expression patterns of genes encoding for proteins strongly involved in these pathways. We believe that characterization of the functionality (e.g. mucosal gene expression profiling) of these pathways could become a way to classify patients for therapeutic decision-making. Therefore, we used the curated Reactome Database to generate lists of genes/proteins that have been described to play a role in to either ER stress or autophagy. Comparisons were made between control individuals, IBD patients with (in)active disease and IFX-responding and non-responding patients.

In this study we show that several ER stress/UPR genes (*KDELR3*, *XBP1*, *DNAJB9*, *SSR1*, *HSP90B1*, *PDIA5, HYOU1, DNAJB11, HSPA5, SYVN1, FKBP14, SEC61A1, CALR, PDIA6* and *WFS1*) are upregulated in the colonic mucosa of patients with active disease. These observed differences were comparable between UC and CD patients and the reduced significance in the comparisons with the CD group is most likely due to the lower sample size (8 CD vs. 74 UC patients). Some genes remained upregulated in patients with inactive disease, however this was only significant (*vs.* control colons) in both cohorts for *KDELR3* and *XBP1*. Nevertheless, this indicates that some molecular players of the ER stress pathway remain active even when there are no overt signs of inflammation, but could possibly trigger disease relapse. Another interesting finding was that *DNAJB9, XBP1, HSP90B1, FKBP14, SRPR, WFS1* and *HERPUD1* were upregulated in patients who will not respond to IFX therapy when compared to patients responding to IFX.

KDEL (Lys-Asp-Glu-Leu) Endoplasmic Reticulum Protein Retention Receptor 3 (KDELR3) is a seven transmembrane spanning receptor that is responsible for anterograde transport of soluble components (such as chaperones) between the ER and the Golgi apparatus[26]. X-binding protein 1 (XBP1) has already been described in chapter 4 since its respective gene is one of the IBD-associated genes that was included in the analysis. In short, *XBP1* mRNA is spliced by the ER stress sensor IRE1α (after BIP dissociation) to form spliced *XBP1* (*sXBP1*) which is subsequently translated into a transcription factor for ER stress response genes in order to reduce ER stress[27]. Therefore, when confirming these microarray data with qRT-PCR, we should use primers that distinguish unspliced XBP1 mRNA from the spliced form as the ratio between both forms has already proven to be a good indicator of UPR activity[28, 29]. DnaJ

Heat Shock Protein Family (Hsp40) Member B9 & B11 (DNAJB9 & DNAJB11), Signal Sequence Receptor Subunit 1 (SSR1), Heat Shock Protein 90 Beta Family Member 1 (HSP90B1), Protein Disulfide Isomerase Family A Member 5 & 6 (PDIA5 & PDIA6), Hypoxia Up-Regulated 1 (HYOU1), Heat Shock Protein Family A (Hsp70) Member 5/Glucose-Regulated Protein, 78kDa (HSPA5/BIP) and FK506 Binding Protein 14 (FKBP14) all have protein stabilizing and/or protein folding functions and their upregulation might therefore be a direct consequence of increased ER stress (signaling)[30-35]. Synoviolin 1 (SYVN1) and Homocysteine Inducible ER Protein With Ubiquitin Like Domain 1 (HERPUD1) play a role in ER-associated degradation (ERAD) for the degradation of misfolded ER-proteins which is also a direct consequence of elevated stress in the ER[36-38]. Sec61 Translocon Alpha 1 Subunit (SEC61A1) and SRP Receptor Alpha Subunit (SRPR) are involved in handling secretory and transmembrane proteins in the ER (and Ca^{2+}-homeostasis in the case of SEC61A1) [39, 40]. Calreticulin (CALR) is u multifunctional protein as it is necessary for Ca^{2+}-storage in the ER but also has chaperone-like functions[41]. Wolframin ER Transmembrane Glycoprotein (WFS1) also is a ER stress response gene that encodes for another multifunctional protein that is part of the ERAD system, regulates ER Ca^{2+}-homeostasis, and suppresses prolonged ATF6α activation[42, 43].

In general, we can conclude that the observed upregulation of ER stress machinery components and response genes in active IBD is in line with current literature. A latent elevation of *KDELR3* and *XBP1* in patients with inactive disease or after successful infliximab therapy might indicate slumbering ER stress in patients who show no clear signs of intestinal inflammation. Finally, the association between *DNAJB9, XBP1, HSP90B1, FKBP14, SRPR, WFS1* and *HERPUD1* expression and non-response indicates that increased ER stress at baseline could signify a risk factor for unsuccessful treatment with anti-TNF agents. Our group already discovered such a predictive genome wide transcriptional signature in this cohort with strong selectivity and specificity. Since our results were derived from the same cohort, including these ER stress genes in the signature by *Arijs et al.* will therefore not increase its performance[24, 25]. However, we do believe that the expression levels of these genes could, on the other hand, support the use of ER stress-reducing agents such as TUDCA and 4-PBA as an add-on treatment in these patients[14, 16-19, 44].

Besides ER stress, we also investigated how autophagy genes were expressed in the same cohorts. The most profoundly upregulated gene in patients with active colonic disease was

Leucine Rich Repeat Kinase 2 (*LRRK2*). This partially confirms a previous study by Gardet *et al.* where the authors also found this association, however only in CD patients and not in the UC group[45]. *LRRK2* is an IBD-associated gene and is therefore also included in the functional analysis described in chapter 4. It encodes for a multi-domain protein that acts as a positive regulator of autophagy through the AMP-activated protein kinase (AMPK) signaling pathway, which is a possible pathophysiologic mechanism in Parkinson's disease. However, in the context of IBD it could also act independent from autophagy: *LRRK2*-deficiency leads to exacerbation of experimental murine colitis through loss of its inhibitory effect on Nuclear factor of activated T-cells (NFAT1) which is independent from LRRK2's kinase activity [46-48]. Regardless of the exact mechanism, this protein seems necessary for maintaining intestinal homeostasis during times of stress and our results indicate a strong activation of this anti-inflammatory mechanism, which appears to be insufficient to prevent or reduce intestinal inflammation in IBD.

In the same analysis we also found 7 significantly downregulated genes in patients with active IBD. Protein Kinase AMP-Activated Non-Catalytic Subunit Beta 2 (PRKAB2) is a positive regulator and a subunit of AMPK that can stimulate autophagy through the AMPK/mTOR/ULK1 pathway[49]. Autophagy Related 14 (ATG14/KIAA0831/BARKOR) is an essential autophagy-specific component and regulator of the phosphatidylinositol (PtdIns) 3-kinase complex which targets the complex to the site of autophagosome formation but also is involved in autophagosome/endolysosome fusion and autophagy-dependent beclin-1 phosphorylation[50-56]. Autophagy Related 4D Cysteine Peptidase (ATG4D) and other ATG4 family members are essential for the (de)conjugation of GABA Type A Receptor Associated Protein Like 1 (GABARAPL1/ATG8) to phosphatidylethanolamine (PE) during autophagosome formation but is also shown to be incorporated into the mitochondrial matrix which can induce ROS-generation and cell death[57, 58]. Microtubule Associated Protein 1 Light Chain 3 Alpha (MAP1LC3A/LC3) belongs to the ATG8-proteins and thus is essential for autophagosome maturation. After conjugation to PE, it is recruited to the autophagosome[56]. Myotubularin Related Protein 3 (MTMR3) is a phosphatase that can dephosphorylate PtdIns 3-phosphate (PtdIns3P) and this increased PtdIns3P can act as a negative regulator of autophagy, a process which has been shown to occur in response to PRR-mediated signals[59, 60]. This can eventually cause to a loss of dampening of NFκB and inflammasome signaling leading to inflammation.

Charged Multivesicular Body Protein 2A & 4B (CHMP4B & CHMP2A) are part of the Endosomal Sorting Complex Required for Transport (ESCRT) III complex that is involved in multivesicular body formation which is required for efficient autophagic degradation of protein aggregates[61, 62]. In summary, all of these downregulated genes, except MTMR3, encode for proteins that will generally stimulate autophagy. Their downregulation is thus in line with current literature where it has been shown that reduced or defective autophagy can lead to intestinal inflammation and IBD. MTMR3 seems to be the odd man out but its downregulation during active disease could lead to an increased responsiveness to microbial patterns which fits the consensus that IBD is an exaggerated immune response towards intestinal microbes.

The fact that *MAP1LC3A* and *CHMP4B* are still downregulated in patients with inactive IBD (eg. after successful IFX-treatment) indicates – similar to the findings observed in ER stress – that not all components of the autophagy machinery are restored to their normal levels. These could contribute to new flares, possibly via inflammasome- or ER stress- dependent mechanisms. Pharmacologic induction of autophagy might therefore be an interesting approach for maintaining an inactive disease state, especially in patients who have a transcriptional signature of reduced autophagy.

Finally, we found that *LRRK2* is significantly downregulated at baseline in patients that will not respond to IFX. Since LRRK2 is a positive regulator of autophagy and LRRK2 KO leads to increased colitis susceptibility in mice, its downregulation in this specific subgroup of patients indicates that reduced or defective autophagy in the intestinal mucosa might be a risk factor for unsuccessful anti-TNF therapy. Also here, these expression profiles could be used for therapeutic decision-making and autophagic-inducers such as sirolimus should be properly studied in IBD treatment[10-13].

It is important to mention that the current analysis remains explorative and confirmation with quantitative RT-PCR and preferably also at the protein level is necessary. For example, some upregulated genes, such as *SEC61A1*, encode for proteins that are part of multimeric complexes, therefore their upregulation doesn't necessarily imply a functional effect. Furthermore, as some of these genes encode multifunctional proteins (eg. *CALR* and *LRRK2*) it remains unsure which function could be affected stressing the need for confirmation at a functional level. Second, in this type of studies the question remains whether the

up/downregultation of specific genes is a cause or rather a consequence of the disease. The answer probably goes both ways as some of these proteins are part of an inflammatory reaction but defects in these pathways have also been shown to cause (intestinal) inflammation itself.

In conclusion, we could show that there is a link between the disease status in IBD and ER stress/autophagy. The microarray analysis of autophagy genes revealed one striking difference when compared to the ER stress related genes. Most autophagy genes were downregulated in patients with active disease whereas the ER stress genes were upregulated in this patient group, corresponding to current literature and insights into the mechanisms driving or triggering disease. Differences in ER stress and/or autophagy gene expression could help in understanding (lack of) response to anti-TNF agents and eventually be used for predicting the response to therapy and to select possible ER stress reducing or autophagy inducing (add-on) therapies.

5. Supplementary material

5.1. Filtering of ER stress genes

From the Reactome database we obtained a list of 94 ER stress genes of which 90 were represented on the Affymetrix GeneChip® Human Gene 1.0 ST array platform and corresponded to 95 probesets. On the Affymetrix Human Genome U133 Plus 2.0 array, 89 out of 94 ER stress genes were represented by 247 probesets. After selecting and retaining the strongest (top-10) upregulated and downregulated genes from every comparison, 43 genes (47 probesets) remained in cohort 1 and 45 genes (109 probesets) remained in cohort 2. The majority of these genes were shared between both analyses. For some genes, the results were inconsistent when comparing both cohorts and were therefore filtered out in order to retain genes that were disregulated in more than one cohort population. This led to a truncated list of 23 ER stress genes.

5.2. Filtering of autophagy genes

From the Reactome database we obtained a list of 69 autophagy genes which were all represented on the Affymetrix GeneChip® Human Gene 1.0 ST array platform and corresponded to 75 probesets. All 69 autophagy genes were also represented on the Affymetrix Human Genome U133 Plus 2.0 array and were represented by 161 probesets. After selecting and retaining the strongest (top-10) upregulated and downregulated genes from every comparison, 38 genes (42 probesets) remained in cohort 1 and 34 genes (97 probesets) remained in cohort 2. Again, after filtering out genes that were only present in a single cohort or showed inconsistent results between both cohorts, we obtained a truncated list of 14 autophagy genes.

5.3. Supplementary tables

Supplementary table 1: ER stress genes under investigation in this study.

ACADVL	*CUL7*	*EIF2S1*	*FKBP14*	*KLHDC3*	*PLA2G4B*	*SRPR*	*YIF1A*
ADD1	*CXXC1*	*ERN1*	*GFPT1*	*LMNA*	*PPP2R5B*	*SRPRB*	*ZBTB17*
ARFGAP1	*DCP2*	*EXOSC1*	*GOSR2*	*MBTPS1*	*PREB*	*SSR1*	
ASNA1	*DCTN1*	*EXOSC2*	*GSK3A*	*MBTPS2*	*SEC31A*	*SULT1A3*	
ASNS	*DDIT3*	*EXOSC3*	*HDGF*	*MYDGF*	*SEC61A1*	*SYVN1*	
ATF3	*DDX11*	*EXOSC4*	*HERPUD1*	*NFYA*	*SEC61A2*	*TATDN2*	
ATF4	*DIS3*	*EXOSC5*	*HSP90B1*	*NFYB*	*SEC61B*	*TLN1*	
ATF6	*DNAJB11*	*EXOSC6*	*HSPA5*	*NFYC*	*SEC61G*	*TPP1*	
ATF6B	*DNAJB9*	*EXOSC7*	*HYOU1*	*ORMDL3*	*SEC62*	*TSPYL2*	
ATP6V0D1	*DNAJC3*	*EXOSC8*	*IGFBP1*	*PARN*	*SEC63*	*WFS1*	
CALR	*EDEM1*	*EXOSC9*	*KDELR3*	*PDIA5*	*SERP1*	*WIPI1*	
CTDSP2	*EIF2AK3*	*EXTL3*	*KHSRP*	*PDIA6*	*SHC1*	*XBP1*	

Supplementary table 2: Autophagy genes under investigation in this study

AMBRA1	*CHMP4C*	*PIK3R4*
ATG10	*CHMP6*	*PRKAA1*
ATG12	*CHMP7*	*PRKAA2*
ATG14	*DYNLL1*	*PRKAB1*
ATG16L1	*DYNLL2*	*PRKAB2*
ATG3	*GABARAP*	*PRKAG1*
ATG3	*GABARAPL1*	*PRKAG2*
ATG4A	*GABARAPL2*	*PRKAG3*
ATG4B	*GABARAPL3*	*RB1CC1*
ATG4C	*LAMTOR1*	*RHEB*
ATG4D	*LAMTOR2*	*RPTOR*
ATG5	*LAMTOR3*	*RRAGA*
ATG7	*LAMTOR4*	*RRAGB*
ATG9A	*LAMTOR5*	*RRAGC*
ATG9B	*LRRK2*	*RRAGD*
BECN1	*MAP1LC3A*	*TSC1*
BRICD5	*MAP1LC3B2*	*TSC2*
C12orf44	*MAP1LC3C*	*ULK1*
CHMP2A	*MLST8*	*UVRAG*
CHMP2B	*MTMR14*	*WDR45*
CHMP3	*MTMR3*	*WDR45B*
CHMP4A	*MTOR*	*WIPI1*
CHMP4B	*PIK3C3*	*WIPI2*

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CHAPTER 7

CONCLUDING DISCUSSION

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1. General discussion

Despite great advances in the last decades, the treatment of IBD is still far from perfect as there is currently no cure yet. Furthermore, even with the treatments available, a large proportion of patients lose response over time or suffer from inacceptable or severe side effects. In order to help this group of patients, often requiring multiple surgical interventions, there is an unmet need to expand the existing therapeutic array. This is already happening as vedolizumab and, more recently, ustekinumab have been approved by the regulatory authorities and have been introduced in clinical practice. Tofacitinib is the first JAKINIB that has been filed for EMA approval for the treatment of ulcerative colitis and more compounds, such as filgotinib, are in late stage clinical development and will hopefully follow soon. By targeting different (more underlying) inflammatory pathways in therapy refractory patients, it is believed that the total number of patients in remission will increase. However, an expanding number of therapies and therapeutic targets also implies that there is an urgent and increasing need for tools and algorithms that help in therapeutic decision making.

In this PhD thesis the **general aim** was to improve IBD treatment at two fronts by, first of all, contributing to the development and potential approval of a novel therapeutic compound (**CHAPTER 3**) and second, by exploring ways to discriminate patients based on the present underlying pathways such as ER stress, autophagy and inflammasome signaling (**CHAPTER 4-6**). We developed a method to culture patient-derived IECs to characterize patient-specific defects in the epithelium at a functional level. We used this model to translate the genetic risk in ER stress and autophagy into functional ER stress readouts (**CHAPTER 4**). Furthermore, we investigated mucosal gene expression of inflammasome sensor genes (**CHAPTER 5**) and ER stress/autophagy genes (**CHAPTER 6**) in two well-characterized patient-control cohorts.

The **first aim** of this project was to assess the efficacy of filgotinib or GLPG0634, an oral JAK-1-specific small molecule inhibitor developed by Galapagos N.V. for the treatment of IBD and RA[1, 2]. This study was part of a, multi-center, scientific project. An extensive *in vivo* mouse study had already been performed at Galapagos where it was shown that filgotinib effectively reduced chronic DSS-induced murine colitis[3]. Nevertheless, the translational value of a single *in vivo* IBD model has always been a subject of discussion as none of the existing models

perfectly resembles human IBD. However, this issue can be overcome by using multiple different animal models[4]. The next step was therefore to confirm the findings from the chronic DSS model in an alternative murine IBD model with a different mode of action at a different research facility (**CHAPTER 3**). The (chronic) DSS model is characterized by an UC-like phenotype which is initiated by chemical destruction of the epithelial barrier followed by dissemination of luminal microbes and their antigens to the lamina propria. It is commonly used because it is a relatively cheap, easy to perform and a very reproducible experimental model[5]. Furthermore, it is characterized by a fast disease onset which limits working hours but, more importantly, it also limits the total time mice suffer from the consequences of intestinal inflammation. As an alternative, we chose the T cell transfer model to confirm the efficacy of filgotinib in IBD. In contrast to the DSS model, it is characterized by a more CD-like phenotype with transmural inflammation in both the small and large intestine. This model is established by injecting the CD4CD45RB^{hi} T cells into the peritoneum of SCID mice. This specific T cell fraction does not contain regulatory T cells and this imbalance between regulatory and effector cells will lead to intestinal inflammation[6]. Despite a relatively good resemblance to human CD, it is a very challenging model characterized by variation in disease severity and time of disease onset. Other disadvantages in comparison to the DSS model are the long duration of the experiment, higher costs and the use of donor mice. Because of the well-known difficulties with this model, we first conducted a pilot experiment and optimized the experimental protocol at our lab. Indeed, we observed great variation in disease severity within a single experiment and differences in disease onset between experiments. In order to reduce the variable disease severity in the final experiment, we included an additional marker for T cell sorting (CD62L) which should lead to the isolation of gut-homing T cells. Furthermore, we increased the number of mice per group and started treating the mice at a time point where we did not observe signs of colitis in the previous experiments. In this final experiment we were able to show that filgotinib, at a daily dose of 30 mg/kg reduced colitis in CD4^{+}CD25^{-} CD45RB^{hi}CD62L^{+}-reconstituted SCID mice when compared to their vehicle-treated littermates. There were significant differences in body weight, macroscopic and histological scores and (although non-significant) a trend for a lower DAI in the filgotinib treatment group. These data, together with the results obtained in the chronic DSS model[3] and data from human intestinal biopsies, supported the use of filgotinib for the treatment of CD and UC. Filgotinib is currently in phase III for the treatment of both CD (DIVERSITY) and UC (SELECTION, phase IIb/III) and

these studies are supported by promising results obtained in the phase II FITZROY trial in patients with moderate-to-severe Crohn's disease[7]. As presented at the latest European IBD conference (ECCO), it was shown that filgotinib demonstrated induction of clinical, histological and biological responses and that clinical remission is associated to reduced pSTAT3-levels[7, 8]. Besides DIVERSITY and SELECTION, two additional phase II trials were initiated in march 2017 for the treatment of small bowel and fistulizing CD (NCT03046056 and NCT03077412, respectively). The most recent available clinical trial data and the fact that tofacitinib has been filed at the EMA, indicate that filgotinib has a good chance to become the second JAKINIB (and the first JAK-1 specific inhibitor) in the therapeutic armamentarium for IBD[7-11]. The class of small molecule compounds has two major benefits over all biologicals: first, its oral administration should be more comfortable thereby leading to better acceptance by patients. Secondly, antibody-based therapies are easily recognized by the patient's immune system. Even fully humanized antibodies such as adalimumab, trigger various forms of hypersensitivity ranging from local injection site reactions to more generalized infusion reactions to even anaphylactic shock. These events are not expected to occur after administration of small molecule compounds such as filgotinib[12]. It is currently too early to say exactly how filgotinib and other JAKINIBs will fit in the classic treatment schedule. In RA, JAK inhibitors have been investigated as an add-on therapy in patients who do not respond to MTX[2] and the FITZROY trial showed that the response rate in anti-TNF naïve patients was twice as high as compared to patients who had already received at least one anti-TNF agent[7]. The latter effect does not appear to be specific for JAKINIBs as this was also observed in some of the vedolizumab and ustekinumab trials[11]. However, filgotinib is not the only promising pharmaceutical compound in late stage clinical development and also alternative treatment strategies such as fecal microbiota transfer and dietary interventions are being investigated for their therapeutic potential in IBD. Hence, the number of available therapies and their modes of action will keep on increasing in the future eventually reaching sufficient diversity in therapeutic armamentarium to maximally treat the entire spectrum of IBD[13, 14]. An increased number of therapies will require tools that allow clinicians to make the best therapeutic choices with the highest clinical and economical benefit/risk-ratio for the patient and the healthcare system. A possible approach is to use a combination of genetic, tissue, fecal and serum biomarkers to assess which pathways are defective in a given patient and their contribution to disease. This

could lead towards better therapeutic guidance by targeting defective pathways rather than the inflammatory phenotype that in the end is the consequence of these defects[14].

Therefore, the **second part** of this PhD aimed at characterizing three IBD-associated pathways in IBD patients at a functional (protein) level and at transcriptional level. The **second aim** was to develop a novel *ex vivo* tool that allows the investigation of patient specific effects/defects at the site of the intestinal epithelium. Furthermore, using this model, we tried to translate the patient's genetic risk in ER stress, autophagy and inflammasomes into quantifiable, functional readouts (**CHAPTER 4**). We have established a short-term patient biopsy-derived two-dimensional *ex vivo* intestinal epithelial cell culture system which was adapted from the three-dimensional intestinal organoid system by Sato *et al.*[15, 16]. We used a nearly identical crypt isolation protocol, but instead of growing the cells inside a matrigel environment, we seeded them on top of a collagen coated well surface. This adaptation allows formation and short-term expansion of cell patches that have a clear epithelial phenotype as indicated by high *CK-18* and *CK-20* mRNA expression over time and E-cadherin-positive immunostainings. Furthermore, a positive MUC2-signal in a subset of these cells and the presence of mucus on bright field microscopy point towards differentiation into functional, mucus-producing goblet cells. Finally, the distribution of ZO-1 at the side facing the medium showed that these cells had a normal apical-basolateral polarization. This novel cell culture system has several potential applications such as the investigation of epithelium specific defects in a wide variety of intestinal diseases, personalized drug toxicity screening and co-culturing on transwells with other patient-derived (immune) cell types such as macrophages or dendritic cells[17]. In this study we showed that an increased number of IBD-associated risk alleles in ER stress and autophagy is associated with a more pronounced Tg-induced ER stress response in the intestinal epithelium; a relationship which was the strongest when the genetic risk in both pathways was combined[18]. Of note, we believe that the observed variation in the response to Tg-stimulation is not due to imprinting from the inflammatory environment since all included biopsies were taken from (macroscopically) non-inflamed areas of the colon. Furthermore, our group recently showed that the inflammatory phenotype of colonic organoids is lost when these cells are taken out of their natural environment, even when this was an inflamed region[19]. On the other hand, Hibiya et al. demonstrated that a 60 week long exposure of colonic murine organoids to an inflammatory environment *in vitro* led to long-lasting (11

weeks) alterations in NfκB signaling[20]. Therefore, it cannot be entirely excluded that such imprinting was also present in some of the included biopsy specimens in our study (eg. when these were obtained from healed unaffected areas that have been inflamed in the past) and that this has an influence on the observed BiP-induction levels. Nevertheless, we feel that such epigenetic changes should be taken into account when determining the functionality of a (disease-associated) pathway in a given patient, which is exactly what these functional assays do. In other words, one of the strengths of functional assays is that the generated readouts are more downstream than (epi)genetic or transcriptomic readouts.

Our results indicate that the genomic risk burden results in altered functionality of the UPR. Nevertheless, we believe that such functional patient-specific readouts by themselves could become an essential part of the novel treatment paradigm which will be discussed below. Quantitative ER stress readouts could become of interest when ER stress reducing compounds become available for the treatment of IBD[21-23]. Furthermore, ER stress appears to be involved in fibrosis as Heindryckx *et al.* showed that IRE1α inhibition leads to reduced fibrosis in animal models but also reverses the fibrotic phenotype of patient derived myofibroblasts[24]. Therefore, it would be interesting to investigate whether there is a link between ER stress defects and a fibrotic disease course in IBD. Although there are no pharmacological therapies to treat intestinal fibrosis, some are under investigation such as the locally acting ROCK-inhibitor AMA0825 which prevented and reverted intestinal fibrosis in two mouse models and an *ex vivo* explant model[25]. Before implementing such epithelial assays as a tool for therapeutic decision making into clinical practice, these findings have to be validated in an independent cohort. This confirmatory study should be performed in a prospective manner where the epithelial ER stress response has to be determined before deciding to apply an additional ER stress reducing therapy. We do realize that this approach has several pitfalls. For example, IBD patients do not always undergo an endoscopy when they visit the hospital and planning an endoscopic investigation for merely therapeutic decision making will probably be considered as being too invasive. Therefore, it will be interesting to investigate whether the readouts obtained in IECs can also be obtained in more accessible patient samples such as blood, feces or even saliva. Moreover, it takes one week before the epithelial ER stress status of a patient is known implying that patients will have to make an additional consultation to have their therapy adjusted. Finally, it must be mentioned that we only succeeded to

efficiently grow IEC cultures from non-inflamed areas in the colon and therefore patients with pancolitis will not be suited for this approach. Nevertheless, we believe that applying this technique around the time of diagnosis will provide a more extensive mechanistic and molecular signature of the patient. Furthermore, it also takes a week or more to have the results from other markers such as thiopurine methyltransferase (TPMT) and vaccination status in the blood, which need to be performed anyway. If around this time the biopsies could be tested for main underlying pathways, then this could help in deciding which drug(s) to give. Most likely, combining therapies targeting different (deficient) pathways may result in higher and better outcomes than what is observed currently with biologicals (30-40% remission with mucosal healing at most).

Besides the ER stress readouts that were described in **CHAPTER 4**, we also obtained functional readouts for the quantification of the autophagic flux by measuring the accumulation of the autophagic cargo marker P62 after chloroquine treatment. In contrast to the functional ER stress readouts, we did not observe any significant difference in P62 accumulation when comparing genetically distinct patients. A possible explanation could be that the investigated risk alleles do not functionally affect autophagy in a profound manner. However, this would be contradictory to the many functional studies investigating the ATG16L1T300A variant[26-29]. Other possible explanations could be that this genetic effect is simply not present in the colonic epithelial compartment or that P62 is not the best marker to investigate the effect of genetic risk variants on autophagy in this setting. Finally, we also aimed to investigate the effect of the genetic risk on inflammasome activation by measuring the amount of secreted IL-18 in the cell culture supernatant. Unfortunately, read-outs were often below the detection limit which led to inadequate power to detect significant differences. Additionally, we set out to evaluate these pathways at a transcriptional level in the intestinal mucosa of IBD patients and controls.

Although being as invasive as the IEC cultures described in CHAPTER 4, transcriptomic data from endoscopic-derived mucosal biopsies can be obtained much faster than functional readouts in IECs as there is no need for epithelial isolation or cell culture. Furthermore, instead of focusing on one or a few markers in a certain pathway, these techniques allow simultaneous analysis of the expression of all involved genes. Our **third and final aim** was therefore to investigate the expression of inflammasome, ER stress and autophagy genes in two whole-

genome gene expression microarray cohorts that had already been well characterized in multiple studies by our group[30-33]. In the first cohort, we compared the colonic mucosal gene expression in patients (CD, UC and grouped together under IBD) with active disease, patients with inactive disease and healthy controls. The second cohort focused on the response to IFX treatment in IBD patients and also allowed comparison to healthy control subjects. In **CHAPTER 5** we used the data from these cohorts to analyze the expression of inflammasome sensor genes that were described in a recent review by Aguilera *et al.*[34]. We found a pronounced increase (>2-fold) in the mRNA expression levels of the DNA-sensing inflammasome sensors *AIM2* and *IFI16* in the inflamed colon of active IBD patients when compared to non-inflamed colon of controls and patients with inactive disease. Furthermore, IFI16 colonic gene expression was, although to a lesser extent, also increased in inactive IBD patients versus control colons. These findings were confirmed in cohort 2 where a significant increase (>5-fold) in expression levels of *AIM2* and *IFI16* in inflamed IBD colon *vs.* control colon was observed. In this cohort we could also show that successful anti-inflammatory treatment with infliximab caused a significant decrease in of *AIM2* and *IFI16* expression when compared to their baseline samples. Although *IFI16* expression decreased after infliximab therapy in IBD responders versus baseline samples, it remained significantly higher after infliximab therapy in IBD responders versus control colons. In contrast with IBD responders, the colonic expression of *AIM2* and *IFI16* remained significantly increased when IFX therapy did not induce remission (non-responders). These data were validated by qRT-PCR, WB and IHC.

It was already known from genetic association and knockout studies that NLRP3 inflammasomes are involved in IBD. In general, this is by far the best studied inflammasome-forming sensor protein. Our data, however, show that the dsDNA-responsive inflammasome sensors AIM2 and IFI16 might also play an important role. Around the same time as our study, another group confirmed, in a much smaller cohort, that *IFI16* is indeed upregulated in inflamed colonic mucosal biopsies from IBD patients in comparison to healthy controls. This upregulation was, at least in part, located in the epithelial cells as shown by immunohistochemistry, confirming our results[35]. More recently, this group has shifted their focus from the actual IFI16 protein to anti-IFI16 antibodies in serum of patients and their value as a biomarker for response to infliximab therapy[36]. Also, AIM2 recently gained more attention and seems to have multiple inflammasome dependent and independent functions that, in the

intestine, converge to intestinal inflammation and cancer. It exerts these effects by influencing antimicrobial peptide production, IEC proliferation, tight junction expression, cell death, barrier function and microbiota composition[37-43]. Both AIM2 and IFI16 or their downstream mediators might thus have some therapeutic potential besides the compounds that target the NLRP3 inflammasome that are currently under investigation[44-47]. Nevertheless, AIM2- and IFI16-selective compounds are not being considered as therapeutic targets since their complex role in IBD needs to be clarified.

Finally, in **CHAPTER 6** we analyzed the expression of ER stress and autophagy genes in the same two cohorts. We used the curated Reactome database to obtain a list of genes that have been involved in one of these two pathways. After applying several filtering steps (FDR < 0.05; top-10 up/downregulated; consistency between cohort 1 and 2), 23 ER stress genes and 14 autophagy genes remained that showed a significant and consistent dysregulation that was associated to disease state. Fifteen ER stress genes were upregulated in the inflamed mucosa of IBD patients when compared to non-inflamed areas and healthy controls, with *KDELR3, XBP1 DNAJB9, HSP90B1* and *CALR* being the strongest upregulated genes. Furthermore, *KDELR3* and *XBP1* were still increased in patients with inactive disease (eg. after successful IFX therapy). *LRRK2* was the only upregulated autophagy gene in patients with active disease together with seven other downregulated genes of which *MAP1LC3A* and *CHMP4B* didn't normalize when disease was under control. The fact that some genes in both pathways remained dysregulated during remission suggests potential underlying mechanisms that can lead to disease relapse. It is possible that (pharmacological) restoration of these pathways could prevent relapse in patients who show such dysregulated gene expression patterns. Finally, we found ER stress and autophagy-specific baseline gene expression signatures in patients who will not respond to IFX therapy. Such predictive transcriptomic signatures have already been discovered in this cohort by our group but their use has so far not made it into clinical practice which might be due to the invasive nature of such biomarkers when compared to serum (CRP) or fecal (calprotectin) biomarkers[32, 33]. Furthermore, we do not believe that including the baseline-dysregulated ER stress and/or autophagy genes in the predictive signature by *Arijs et al.* will increase its performance as we used the same cohort to obtain our results[32, 33]. Despite being invasive, we do feel that until there are no alternative methods to detect mucosal defects in specific pathways, the mucosal expression levels of genes in the ER

stress or autophagy pathways could support the use of (add-on) therapies such as TUDCA or sirolimus.

We believe that the best predictive models will arise from multi-omics approaches that combine genomic, transcriptomic and/or proteomic information in order to give a clear indication whether a certain pathway (eg. autophagy) is perturbed in a given patient. There is already some evidence that a functional autophagy pathway is needed for an adequate response to anti-TNF as autophagy is upregulated in anti-TNF induced immunosuppressive macrophages and that functional autophagy is necessary for this specific conversion[48, 49].

General conclusion

This PhD thesis added potential improvements to IBD therapy by providing additional evidence for the acceptance of filgotinib for the treatment of IBD by the authorities and by exploring ways to identify and characterize underlying disease-associated pathways in the intestinal mucosa. This pathways-based approach could guide clinicians in deciding when to give a certain therapy.

2. Future perspectives

I would like to propose some additional experiments that could and should be performed in the future. Since most of the IBD-associated genetic variants that were investigated in **CHAPTER 4** are located outside of exons, one possible functional effect might therefore be altered transcriptional regulation. Therefore, it would be very interesting to perform eQTL analysis with these SNPs in mucosal biopsies, but even more so in sorted cells of the same type (eg. separated IECs, T cells, macrophages). Also, the statements made in **CHAPTER 6** are largely based on microarray data and will need to be confirmed by other techniques and preferably also by WB and IHC to determine the (sub)cellular location of interesting proteins such as XBP1, KDELR3, and LRRK2. We are also convinced that the epithelial cell culture system that was developed and described in **CHAPTER 4** has a lot of potential applications. Given the clear involvement of the intestinal microbiota in IBD, a possible future approach might be to co-culture biopsy-derived epithelial cells with specific bacterial strains to study patient-specific microbe-host interactions.

Finally, it is important to reflect how this thesis focuses on the evolving landscape of IBD management: filgotinib and other JAKINIBs may become important compounds for treating IBD but also other inflammatory disorders such as RA. An increasing number of therapies and therapeutic targets for the treatment of complex, multifactorial diseases demands a radical change of the current treatment paradigm. Our group has the ambition to play a leading role in this effort as illustrated by the second part of this thesis manuscript which fits into a larger ERC-granted project where IBD UZLeuven wants to develop and validate the Crohn's and Ulcerative Colitis Characterization and Intervention (CrUCCial) index. In the CrUCCial project we aim to quantify different potential pathophysiologic players such as ER stress, autophagy, barrier dysfunction and dysbiosis in a personalized manner. These readouts will be generated from extensive and diverse sampling starting at the time of diagnosis. Thanks to this integrative multi-omics approach, we will be able to identify the proportional contribution of the different mechanisms in a given patient. Therefore, in this PhD thesis, we developed a method to quantify patient specific epithelial defects in pathophysiologic pathways and have initially validated this concept by demonstrating a link between genetic mutations and functional defects in ER stress. This is a possible readout that can now be generated from patient biopsies and that will be implemented in the CrUCCial index.

We believe that also transcriptomics could be a valuable tool in this light as they form the basis for downstream functional effects. In this PhD I chose to work with the existing microarray cohorts because I felt that there was still some unexplored areas in these datasets. However, we are aware that more sensitive techniques, such as RNA sequencing, are available. This is part of new ongoing projects in our lab. Nevertheless, our microarray findings still indicate on the functionality and activation of a certain pathway and which genes to look for when assessing the relative contribution of inflammasomes, ER stress and autophagy to IBD.

The CrUCCial index will statistically combine a variety of different readouts in a semi-quantitative way into a multi-component index that will be used to tailor therapies to the specific needs of the patient. This index will be tested for its ability to predict clinical outcomes such as time to first surgery, stricturing disease behaviour, penetrating disease course, etc. The obtained findings from these discovery cohorts and the use of the CrUCCial index to guide therapeutic decision making in the clinic will eventually be evaluated in prospective cohorts.

Our integrative multi-omics and multi-pathway approach should lead to profound alterations in the future treatment paradigm of IBD and other complex, multifactorial conditions by treating specific defective pathways rather than a general disease phenotype.

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SUMMARIES

1. English summary

Inflammatory bowel diseases (IBD), with Crohn's disease (CD) and ulcerative colitis (UC) as the two most prevalent phenotypes, span a disease spectrum of lifelong, idiopathic conditions characterized by chronic inflammation of the gut with diarrhea and abdominal pain. Although its exact pathophysiology remains elusive, IBD is believed to be caused by an aberrant mucosal immune responses towards intestinal microorganisms in genetically predisposed individuals. During the last years, new pathophysiologic pathways have been uncovered and since there still is an unmet therapeutic need in IBD, this knowledge is currently being applied by pharmaceutical companies to develop compounds that target these pathways. Therefore, in the first part of this project we aimed to contribute to the therapeutic development for IBD patients by performing a preclinical efficacy experiment in mice for a new experimental JAK1-specific inhibitor (GLPG0634/filgotinib) that has been developed by Galapagos NV (**CHAPTER 3**). Since the efficacy of filgotinib was already shown in a chemically induced murine colitis model (DSS), we performed these experiments in the T cell transfer model of colitis which is an adaptive/T cell-driven model. Daily oral administration of filgotinib resulted in a significant reduction in body weight loss and histological inflammation scores. Our data, together with the data obtained from the chronic DSS model, provide convincing preclinical evidence for the application of filgotinib in IBD and meanwhile a large phase 3 program is ongoing.

Other new therapeutic strategies, are in the research pipeline or reaching registration. This means that, in the future, the range of therapies will drastically expand. Furthermore, a well-known characteristic of IBD is the large inter-patient variability in disease course and severity, which has consequences for the type, the dose and frequency of treatment. We believe that the IBD treatment paradigm should change towards a more personalized approach that is based on targeting the underlying pathophysiologic pathways that drive the disease in a given patient. The second part of this PhD project investigated the contribution of three IBD-associated pathways (ER stress, autophagy and inflammasomes) in specific patient-subgroups at different levels. First of all, we developed a novel cell culture system that allows short term expansion of patient/biopsy-derived intestinal epithelial monolayers (**CHAPTER 4**). We confirmed that these monolayers had an epithelial character and a normal apical-basolateral polarization. In this model we showed that the number of risk alleles in ER stress and/or

autophagy leads to an increased BIP induction after thapsigargin treatment indicating functional alterations in the epithelial ER stress response. These readouts might in the future be used as an indication for ER stress reducing therapies such as TUDCA. Furthermore, we believe that this patient-derived intestinal epithelial cell culture approach has more potential applications such as co-culturing with other cell types and/or investigating patient-specific responses to pharmaceuticals or even microbes.

Finally, we investigated the inflammasome, ER stress and autophagy at a transcriptional level in the mucosa of patients and healthy controls who were included in two well-characterized whole genome gene expression microarray cohorts. We found a strong upregulation of two dsDNA-responsive inflammasome sensors *AIM2* and *IFI16* in patients with active disease when compared to patients with inactive disease or healthy controls (**CHAPTER 5**). We are one of the first groups to identify the potential involvement of both inflammasome sensors which are now subject of investigation as a disease activity marker (IFI16) and a key player in intestinal homeostasis (AIM2). We also found a strong upregulation of several ER stress genes and a downregulation of autophagy genes that was associated with increased disease activity (**CHAPTER 6**). When disease was controlled normalization was observed for most of these genes, yet some (*KDELR3*, *XBP1, MAP1LC3A* and *CHMP4B*) remained dysregulated, indicating that these pathways are not entirely restored to their normal levels upon remission. Finally, we found a gene expression signature in these pathways that could aid in predicting the response to anti-TNF treatment caused by an altered ER stress or autophagy status. Although preliminary, these data hint towards the application of ER stress reducing or autophagy stimulating therapy in patients who show, already at baseline, dysregulated gene expression patterns.

In conclusion, the promising filgotinib data demonstrate that we live in a time of increasing therapeutic diversity for IBD. In this PhD thesis we furthermore set the first steps towards a personalized pathway-based approach for treating this multifactorial disease spectrum.

2. Nederlandstalige samenvatting

Inflammatoire darmziekten (IBD), met de ziekte van Crohn en colitis ulcerosa als de twee meest voorkomende fenotypes, zijn levenslange, idiopathische aandoeningen die worden gekenmerkt door chronische ontsteking van de darm met diarree en buikpijn. Hoewel de exacte pathofysiologie onduidelijk blijft, vermoedt men dat IBD wordt veroorzaakt door een overdreven immuunrespons tegen de intestinale microbiota in genetisch voorbestemde individuen. Tijdens de laatste jaren zijn nieuwe pathofysiologische mechanismen ontdekt die een rol spelen in IBD. Deze kennis wordt ook gebruikt om nieuwe geneesmiddelen te ontwikkelen die ingrijpen op deze nieuwe pathofysiologische mechanismen. In het eerste deel van dit doctoraatsproject hebben we daarom getracht een bijdrage te leveren aan deze therapeutische ontwikkelingen door de werkzaamheid van een JAK1-specifieke inhibitor (GLPG0634/filgotinib) na te gaan in een colitis muismodel (**HOOFDSTUK 3**). Dit molecule werd ontwikkeld door Galapagos NV en de werkzaamheid werd eerder aangetoond in een chronisch DSS muismodel. Bevestiging in een tweede model was echter noodzakelijk. Wij hebben hiervoor gebruik gemaakt van het T cel transfer muismodel en konden aantonen dat dagelijkse orale toediening van filgotinib leidde tot een significante reductie van het verlies aan lichaamsgewicht en histologische ontstekingsscores. Onze data leveren, samen met de gegevens verkregen uit het chronische DSS-model, kwalitatief preklinisch bewijs voor het gebruik van filgotinib in IBD. Een groot klinisch fase 3 programma in IBD patiënten is momenteel aan de gang.

Er zijn nog andere nieuwe therapieën die zich in vergevorderde onderzoeksfasen bevinden. Dit betekent dat het aantal mogelijke therapieën voor IBD verwacht wordt verder toe te nemen in de toekomst. Bovendien is het een welgekend fenomeen dat IBD patiënten een grote diversiteit vertonen in het ziekteverloop en de ernst van de ziekte en dit heeft gevolgen voor het type, de dosis en de frequentie van de behandeling. Wij geloven dat het IBD-behandelingsparadigma moet evolueren naar een meer gepersonaliseerde aanpak die gebaseerd is op het identificeren en behandelen van de onderliggende pathofysiologische mechanismen die de ziekte veroorzaken. In het tweede deel van dit doctoraatsproject hebben we daarom de bijdrage van drie IBD-geassocieerde mechanismen (ER stress, autofagie en het inflammasoom) onderzocht in specifieke patiënt-subgroepen en op verschillende niveaus. Allereerst hebben we een nieuw celcultuur systeem ontwikkeld dat ons toeliet om

darmepitheelcellen in kweek te brengen die werden geïsoleerd uit endoscopische darmmucosa biopten (**HOOFDSTUK 4**). We konden aantonen dat deze cellen een epitheliaal fenotype hadden met een normale polarisatie. Vervolgens hebben we dit model gebruikt om aan te tonen dat het aantal risico-allelen in ER stress en/of autofagie leidt tot een verhoogde BIP inductie na de behandeling met thapsigargine. Een verhoogd genetisch risico leidt dus tot wijzigingen in de epitheliale ER stress respons en deze aanpak zou in de toekomst gebruikt kunnen worden als indicatie voor het opstarten van ER stress-reducerende therapieën zoals TUDCA. Bovendien kunnen deze patiënt-afgeleide epitheliale culturen andere potentiële toepassingen hebben, zoals co-culturen met andere celtypes en/of het onderzoeken van patiënt-specifieke reacties op farmaceutische verbindingen of zelfs bacteriën.

Tenslotte hebben we het inflammasoom, ER stress en autofagie op transcriptieniveau onderzocht in de colonmucosa van patiënten en gezonde controles in twee grondig gekarakteriseerde genexpressie microarray cohorten. We vonden een sterke toename van twee dsDNA-responsieve inflammasoomsensoren *AIM2* en *IFI16* bij patiënten met actieve ziekte in vergelijking met patiënten met inactieve ziekte of gezonde controles (**HOOFDSTUK 5**). Hierdoor zijn we één van de eerste groepen die AIM2 en IFI16 naar voren schuiven als potentiële actoren in IBD. AIM2 wordt nu bestudeerd omwille van zijn veelzijdige rol in darmhomeostase terwijl IFI16 onderzocht wordt als een mogelijke ziektemerker in IBD. In dezelfde cohorten konden we tevens een sterke toename van meerdere ER stress genen en een afname van autofagie genen waarnemen in patiënten met actieve ziekte (**HOOFDSTUK 6**). De meeste van deze genen normaliseerden weer wanneer de ziekte onder controle was met uitzondering van *KDELR3*, *XBP1*, *MAP1LC3A* en *CHMP4B*, wat aangeeft dat beide mechanismen, ondanks een normaal endoscopisch beeld, niet volledig herstellen. Tot slot vonden we een afwijkend genexpressiepatroon in enkele genen die mogelijks kunnen bijdragen aan het voorspellen van de respons op anti-TNF-behandeling. Hoewel deze data preliminair zijn, geven ze toch een indicatie voor het gebruik van ER stress vermindering of autofagie stimulatie therapieën bij patiënten met een afwijkend genexpressiepatroon.

Samenvattend denken we dat we in deze doctoraatsthesis de eerste stappen hebben gezet in het sturen van een gepersonaliseerde behandeling van dit multifactoriële ziektespectrum.

3. Popular summary

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC) are lifelong diseases of unknown cause characterized by inflammation of the intestine with diarrhea and belly pain. Although it remains unknown how these diseases arise, IBD are believed to be caused by an exaggerated immune responses towards intestinal microbes in people who have a genetic predisposition. During the last years, new disease mechanisms have been discovered while there is still a large proportion of patients that cannot be helped with the current therapies. Pharmaceutical companies are therefore developing drugs that act on these newly discovered disease mechanisms. In the first part of this PhD project we aimed to contribute to the therapeutic development for IBD patients by testing the efficacy of GLPG0634/filgotinib, which specifically blocks the inflammatory signaling molecule JAK1, in a mouse model of colitis (**CHAPTER 3**). In this model we showed that daily oral administration of filgotinib reduced intestinal inflammation as indicated by a strong reduction in body weight loss and this anti-inflammatory effect was also seen at a microscopic level. Our data, provide convincing evidence for the therapeutic application of filgotinib in IBD, meanwhile a large trial in IBD patients is ongoing.

Other new drugs are under investigation and are close to becoming available for IBD patients. This means that, in the future, the number of therapies will further increase. Furthermore, a well-known characteristic of IBD is the large diversity between patients in disease course and severity, which has consequences for the type, the dose and frequency of treatment. We believe that the current way of IBD treatment should change towards a more personalized approach that is based on targeting the underlying disease mechanisms that cause the disease in a given patient. The second part of this PhD project investigated the contribution of three such disease mechanisms that play a role in IBD, namely "ER stress", "autophagy" and "inflammasomes". First, we developed a method to grow gut cells (in the lab) that were taken from patients during endoscopy (**CHAPTER 4**). We confirmed that these so called cell cultures were indeed epithelial cells and that these cells had the same orientation as they have when they are still in the gut. With this method, we showed that patients who have an increased amount of ER stress- and/or autophagy-related genetic risk factors show problems in the ER stress mechanism. This approach can thus be used to identify patients that would benefit from therapies that reduce ER stress such as TUDCA. Furthermore, we believe that this patient-

derived gut epithelial cell culture method has more potential applications such as growing them together with other cell types and/or investigating the interaction between these cells and drugs or even microbes.

Finally, we investigated the inflammasome, ER stress and autophagy mechanisms at the level of gene expression in the intestine of patients and healthy controls. We found a strong increase of the inflammasome genes *AIM2* and *IFI16* in patients with active disease when compared to patients with inactive disease or healthy controls (**CHAPTER 5**). We also found a strong increase of several ER stress genes and a decrease of autophagy genes in patients with active disease (**CHAPTER 6**). Most of these genes returned to their normal levels in patients with inactive disease, yet some (*KDELR3*, *XBP1, MAP1LC3A* and *CHMP4B*) remained dysregulated even when disease was controlled indicating that both pathways are not entirely restored.

In conclusion, the promising filgotinib data are a clear example of an increasing number of therapies for IBD in the future. In this PhD thesis we furthermore set the first steps towards a personalized treatment based on the underlying disease mechanisms.

4. Popular summary (Dutch)

Inflammatoire darmziekten (IBD), waaronder de ziekte van Crohn en colitis ulcerosa zijn levenslange aandoeningen van ongekende oorzaak die worden gekenmerkt door chronische ontsteking van de darm met diarree en buikpijn. Hoewel het exacte ontstaansmechanisme van deze ziekten onduidelijk blijft, vermoedt men dat IBD wordt veroorzaakt door een overdreven immuunrespons tegenover de normale darmbacteriën in personen met een genetische aanleg. Tijdens de laatste jaren heeft men nieuwe ziektemechanismen ontdekt die een rol spelen in IBD. Aangezien niet alle patiënten kunnen geholpen worden met de huidige therapieën, wordt deze kennis gebruikt door farmaceutische bedrijven om nieuwe geneesmiddelen te ontwikkelen die gericht zijn tegen deze recent ontdekte ziektemechanismen. In het eerste deel van dit doctoraatsproject hebben we daarom getracht een bijdrage te leveren aan de ontwikkeling van een nieuw medicijn voor IBD, filgotinib (**HOOFDSTUK 3**). We hebben de werkzaamheid van dit molecule nagegaan in een muismodel dat bepaalde eigenschappen van IBD nabootst en konden hierin aantonen dat de dagelijkse toediening van filgotinib leidde tot een verminderd gewichtsverlies en lagere microscopische ontstekingsscores. Onze data leveren bewijs voor het gebruik van filgotinib in IBD. Een grote klinische studie in IBD patiënten is momenteel aan de gang.

Er zijn nog andere nieuwe therapieën die zich in de laatste onderzoeksfasen bevinden. Dit betekent dat het aantal mogelijke therapieën voor IBD verder zal toenemen in de toekomst. Bovendien is het een welgekend fenomeen dat IBD patiënten een grote diversiteit vertonen in het ziekteverloop en de ernst van de ziekte en dit heeft gevolgen voor het type, de dosis en de frequentie van de behandeling. Wij geloven dat de manier waarop we IBD behandelen moet evolueren naar een meer gepersonaliseerde aanpak die gebaseerd is op het identificeren en behandelen van de onderliggende ziektemechanismen die de ziekte veroorzaken. In het tweede deel van dit doctoraatsproject hebben we daarom de bijdrage van drie zulke ziektemechanismen (ER stress, autofagie en het inflammasoom) onderzocht in specifieke patiënt-subgroepen. Allereerst hebben we een nieuw celcultuur systeem ontwikkeld dat ons toeliet om darmepitheelcellen in kweek te brengen die werden geïsoleerd uit darmbiopten (**HOOFDSTUK 4**). We konden aantonen dat deze cellen inderdaad behoorden tot het juiste (epitheliale) celtype en dat ze normaal georiënteerd waren. Vervolgens hebben we deze zogeheten celculturen gebruikt om aan te tonen dat een toegenomen aantal

genetische risicofactoren in ER stress en/of autofagie leiden tot een gewijzigde ER stress respons in deze cellen. Deze aanpak zou in de toekomst gebruikt kunnen worden als leiddraad voor ER stress-reducerende medicatie zoals TUDCA. Bovendien kunnen deze patiënt-afgeleide celculturen andere potentiële toepassingen hebben, zoals het opkweken samen met andere celtypes en/of het onderzoeken van patiënt-specifieke reacties op medicatie of zelfs bacteriën.

Tenslotte hebben we de activiteit van inflammasoom, ER stress en autofagie genen onderzocht in het darmweefsel van patiënten en gezonde controles. We vonden een sterke toename van de inflammasoom genen *AIM2* en *IFI16* bij patiënten met actieve ziekte in vergelijking met patiënten met inactieve ziekte of gezonde controles (**HOOFDSTUK 5**). Verder konden we tevens een sterke toename van meerdere ER stress genen en een afname van autofagie genen waarnemen in patiënten met actieve ziekte (**HOOFDSTUK 6**). De meeste van deze genen keerden terug naar hun normale niveau wanneer de ziekte weer onder controle was met uitzondering van *KDELR3*, *XBP1*, *MAP1LC3A* en *CHMP4B*. Dit geeft aan dat beide mechanismen, ondanks een normaal endoscopisch beeld, niet volledig herstellen.

Onze veelbelovende data met filgotinib in een colitismodel tonen reeds aan dat we leven in een tijd waarin het aantal therapieën voor IBD nog steeds toeneemt. Verder zetten we in deze doctoraatsthesis de eerste stappen in de richting van een gepersonaliseerde behandeling van dit multifactoriële ziektespectrum.

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PERSONAL CONTRIBUTION

CHAPTER 1-2: All

CHAPTER 3: Experimental design, disease monitoring, sacrifice, scoring, data analysis, manuscript revision

CHAPTER 4: All

CHAPTER 5: Confirmation of microarray data with PCR, WB an IHC, manuscript writing, figure creation

CHAPTER 6-7: All

CONFLICTS OF INTEREST STATEMENT

Wiebe Vanhove has no conflicts of interest

Kris Nys is a MSL for Shire since 2016

Ingrid Arijs is a project manager at the University of Hasselt since 2016

Severine Vermeire reports following conflicts of interest: grant support from Abbvie, MSD and Takeda; lecture and consulting fees from Centocor, MSD, Abbvie, Pfizer, Takeda, Genentech/Roche, Janssen, Mundipharma, Hospira, Celgene and Second Genome. She is a senior clinical investigator for the FWO.

Marc Ferrante reports following conflicts of interest: Grant support from Takeda; lecture and consulting fees from Abbvie, MSD, Takeda, Janssen, Boehringer-Ingelheim, Chiesi, Dr Falk Pharma, Ferring, Mitsubishi Tanabe, Tillots and Zeria. He is a senior clinical investigator for the FWO.

Gert Van Assche reports following conflicts of interest: grant support from Abbvie and MSD; lecture and consulting fees from Abbvie, Ferring, MSD, Takeda and Janssen. He is a senior clinical investigator for the FWO.

All other authors have no additional conflicts of interest regarding this PhD manuscript.

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We now have arrived to the what is maybe the most important (an definitely the most read) part of my PhD where I would like to express my gratitude to all people that have been involved in my scientific career so far.

Before going over to Dutch I would like to thank all the jury members for being here today and for taking the time to critically read and comment on my PhD manuscript.

Professor Kaser, you probably don't know this, but I'm a big fan of the work from your research group. I have read multiple publications with great pleasure and interest, they were always of very high quality and have made a substantial contribution to my knowledge on ER stress and autophagy in the context of IBD. It is therefore a great honor that you were willing to be part of my jury and for that, you have my eternal gratitude. I hope you will enjoy the rest of your short stay at Leuven and wish you a safe trip home tomorrow morning.

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"Sometimes science is a lot more art than science, a lot of people don't get that."
Rick Sanchez

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Zoals gezegd, was het Ingrid die me bij de IBD-groep heeft gehaald en me dan ook voorstelde aan mijn promotor: Professor Vermeire. Beste **Séverine**, bij onze eerste ontmoeting werd mij meteen duidelijk dat je iemand was met enorm veel goede, creatieve ideeën en een duidelijke visie. Nadien leerde ik dat je dit dan ook nog eens wist te combineren met een bijna onmenselijke efficiëntie en fantastische people management skills. Ik heb me de afgelopen vier jaar meermaals afgevraagd of je een toverstaf, kristallen bol of teletijdmachine had, want ik begrijp nog steeds niet hoe jij al die dingen tegelijk aankan. Een andere eigenschap die ik enorm aan je apprecieer is dat ik nooit een echte drempel heb gevoeld om iets aan jou te vragen, ik ben altijd mezelf kunnen blijven en dat vind ik enorm belangrijk. Je stuurt onze groep als de beste en slaagt erin om ieders project in goede banen te leiden, ik prijs me dan ook erg gelukkig dat ik hier deel van uit mocht maken.

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"Planning for failure is even dumber than regular planning."
Rick Sanchez

teambuilding te zetten, ik zal deze leuke laboweekendjes nooit vergeten. Je zorgde er tevens voor dat we tijdens DDW met de hele groep samen in een huisje konden verblijven en was ook steeds van de partij op de buitenlandse etentjes. Dankzij deze initiatieven is de IBD-groep een hechte familie waar iedereen zich thuis voelt, merci. **Gert**(jeuh!), je was dan niet rechtstreeks betrokken bij mijn project, toch ben ik blij dat ik je samen met jouw leuke anekdotes heb leren kennen en af en toe eens de Samson kon uithangen. Ook nog een dikke merci aan jullie alle drie voor de vele biopten die jullie voor mijn project hebben afgenomen!

Dan zou ik nu willen overgaan naar alle leuke collega's binnen de IBD-groep en dat zijn er een heel aantal. Allereerst de mensen die reeds vertrokken zijn. **Nils**, je bent een topkerel die het zeker ver zal schoppen in San Diego, bedankt om ons steeds de weg te wijzen en op sleeptouw te nemen tijdens DDW. **Thomas**, je was een fijne collega, maar het is vooral buiten het werk dat ik je beter heb leren kennen. Jij was altijd degene die knopen doorhakte en de groep mee op sleeptouw kon nemen. Jouw organisatietalent bij het splitten/betalen van de rekening op restaurant wordt trouwens nog steeds gemist. **Dominiek**, bedankt om steeds voor de nodige dosis gezever en schurkenstreken te zorgen, hopelijk kunnen we hier af en toe op de oude markt nog een aantal vervolgen aan brijen. **Karolien**, ik vond het jammer om je te zien vertrekken, maar ben blij dat ik je nog af en toe tegenkom in de gang. **Nikolai**, nog zo iemand die ik liever bij ons had zien blijven, ik kon het altijd goed met je vinden en hoop dat we elkaar nog eens zien met een goeie pint erbij. **Isabelle**, je bent ondertussen zelf professor van je eigen groep(je), toch voelt het voor mij aan alsof je nog steeds bij onze groep hoort. Bedankt voor je hulp, feedback en het beantwoorden van al mijn geneticavragen. **Sare**, ik leerde je kennen tijdens je masterthesis waarin je dankbaar gebruik maakte van onze biopten. Ik ben heel blij dat je nadien ook zo nauw aan onze groep verbonden bent gebleven en wens je veel succes met de rest van je doctoraat. Alle begin is moeilijk, maar we gaan ongetwijfeld nog veel horen van 'The Cleynen-lab'! **Kostas** (aka sports-guy), it was a pleasure to have you in our group and to have our daily football-talks. It is still a mystery to me how you manage to know all the results from every league, you really are a walking/talking encyclopedia when it comes to sports. I enjoyed having you at OHL to support my team or to see the Belgium woman team kick Greece's ass. Thank you for the Olympiakos shirt and scarf, I hope that one day I will be able to join you for one of their home games! I wish you all the best in the US my friend. Also, best of luck to our two Spanish fellows **Triana** and **Aranza**. **Anthony**, het was leuk om u erbij

"Let the world change you and you can change the world"
Ernesto Che Guevara

te hebben op teambuilding, doe dat goed daar in Canada. Aan de twee mensen die bijna terug in Leuven zijn: **Alessia** en **Willem-Jan**, ik hoop dat jullie een leuke tijd hadden in Italië en kijk ernaar uit om jullie binnen enkele maanden terug bij onze groep te hebben. En dan onze meest recente vertrekker: **Manuel**, je was een leuke collega waar ik me goed me heb geamuseerd. Ik ben er helaas niet in geslaagd om je te leren drinken als een echte Belg, maar ik weet niet of dat écht aan mij lag… Op congres hebben we meerdere keren een veel te klein bed moeten delen, maar op 1 nachtje snurken na, had het veel erger gekund ☺. Veel succes met je postdoc in Chicago, dat wordt vast en zeker een fantastische ervaring.

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"Het eten is de belangrijkste maaltijd van de dag."
Wiebe Vanhove

optreden proberen mee te pikken in de toekomst. **Nooshin**, you are a wonderful person who is always willing to help. Thank you for being the friendly, caring person that you are and you for taking care of all the little extra things I asked you during the past years. **Helene**, met jouw aanstekelijke lach was je meteen een aanwinst voor ons team en je slaagt er ook telkens in om een glimlach op mijn gezicht te toveren. Hopelijk blijf je nog lang bij ons! **Tamara**, door omstandigheden heb ik je helaas nooit goed leren kennen. Ik hoop dat je er snel weer bovenop komt zodat je met een glimlach door het leven kan gaan.

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“Ik ben blij dat gij in mijn team zit”
Guido Pallemans

mens heb ik veel bewondering voor jou, ik denk dan ook dat al jouw (toekomstige) patiënten zich enorm gelukkig (zullen) prijzen met zo'n zorgzaam persoon als arts. Als er één persoon is die alles heeft om het te maken als de volgende Leuvense IBD-professor, dan ben jij het wel. **Brecht**, je zit spijtig genoeg niet bij ons in het kantoor, maar ik ben blij dat je er steeds bij bent wanneer we na het werk nog iets gaan doen. Binnenkort zal ik hoogstwaarschijnlijk enkele muismodellen opstarten dus dan zullen we elkaar zeker wat meer zien op de werkvloer. Ik wens je nog veel succes met de ILCs en wens je nog oneindig veel steengoeie Radiohead concerten toe. (Tante) **Sofie**, je bent een fijne collega waar ik het heel goed mee kan vinden, ik ga dan ook blij zijn als je terug uit zwangerschapsverlof bent en er weer bij kan zijn. **Clara** (aka madam kak), ik ben content dat ik ook jouw eerste jaar bij ons nog heb mogen meemaken. Ik hou van je aanstekelijke, energieke, "zonder zeveren-mentaliteit" en ben ervan overtuigd dat je hiermee jouw FMT-project zeker tot een goed einde zal brengen. Ik hoop dat ik Caenepeel et al. in de toekomst nog veel ga mogen citeren! **Kaline**, jou ken ik nog niet zo lang, maar ik merkte op teambuilding meteen op dat je een heel sociaal en behulpzaam persoon bent. Ook op wetenschappelijk vlak ben je ongetwijfeld een aanwinst voor onze groep, ik ben dan ook heel blij dat ik tijdens de komende maanden nog veel met jou ga mogen samenwerken. **Annick** en **Karen**, jullie zijn onze twee nieuwste aanwinsten die ik daardoor helaas nog niet zo goed ken. De eerste maanden met jullie erbij waren alleszins heel aangenaam en ik ben dan ook blij dat ik er minstens nog enkele maanden bij mag doen. Ik wens ook jullie alle succes van de wereld met jullie doctoraat en alles wat erna nog zal volgen.

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"Remember children, there are no stupid questions…
…just stupid people"
Herbert garrison

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Aangezien de IBD-groep valt onder TARGID zijn er ook enkele mensen die ik hier moet bedanken voor de afgelopen vier jaren. Allereerst de PI's die deze unieke werkplek in stand houden en niet vergeten dat teambuildings en kerstfeestjes een belangrijke bijdrage leveren aan de goeie werksfeer. **Ricard** en **Pieter**, maar ook **Hanne**, **An-Sofie** en **Valerie**, bedankt voor jullie hulp en feedback bij de revisie van mijn paper, mede dankzij jullie had ik mijn tweede publicatie vooraleer mijn thesis binnen moest. De PI's zitten dan wel aan het stuur van de TARGID-motor, maar er zijn twee heel bijzondere mensen die er iedere dag opnieuw voor zorgen dat die motor niet meteen ontploft: **Cindy** en **Phyllis**. Dank u om telkens zo snel te reageren wanneer ik weer met een (administratief) probleem zat, maar ook voor de leuke babbeltjes af en toe, jullie zijn fantastisch. Ik heb zeer veel respect voor jullie en ben enorm dankbaar voor wat jullie allemaal doen voor onze groep. Verder nog enkele andere TARGID-mensen die ik hier toch even wil vermelden, aan diegenen die ik hier vergeet: please don't shoot me. **July**, good luck with your new job, I already miss our funny conversations in the hallway or during the after-work occasions. We will probably meet again in the beautiful city of Tienen and when that time comes we should just go for a drink (or six). **Charlotte**, je was een van de eerste mensen op TARGID waar ik een goeie band mee had en waar ik steeds een babbelke mee kon doen. Of je nu jouw doctoraat afmaakt of niet, ik wens je veel succes en plezier met je carrière! **An-Sofie**, we hebben tegelijkertijd de eindspurt naar onze verdediging ingezet waardoor we veel aan elkaar hebben gehad de laatste maanden. Ik ben er zeker van dat je dat fantastisch gaat doen volgende maand. **Seppe**, je hebt het epitheliale celcultuur project op de rails gezet tijdens jouw masterthesis en ik pluk daar vandaag de dag nog steeds de vruchten van. Buiten het werk hebben we een aantal matchen meegepikt aan den Dreef en misschien kom ik ooit eens mee naar Anderlecht (of toch niet, ik supporter niet voor jeanettenploegen, sorry ☺). **Elisa**, it was fun to have you in our group during the UEGW basic science workshop in Amsterdam and I'm happy that I got to know you a little better. I had a lot of fun at Rock Werchter even though we lost each other during the System of a down concert. The same goes for **Alessandra** and I'm pretty sure we'll run into each other at a

"A hungry man, is an angry man"
Robert Nesta (Bob) Marley

(reggae) party or festival. (DJ-)**Jess**, I'm really sad that you had to leave us, but I'm glad we ended in style at the Metafoor. Good luck in Australia and next time you come back to Belgium, the Wolfkes are on me! To those that I didn't mention here personally, please don't feel offended, you are all great and you know that I love you ☺.

Ik heb nu reeds enkele pagina's gevuld met lofzangen over al mijn fantastische (ex-)collega's, maar de voorbereiding van dit doctoraat duurde eigenlijk nog veel langer (en het leven is meer dan werken alleen). Hoog tijd dus om over te gaan naar al mijn vrienden en familie die op één of andere manier een steentje hebben bijgedragen aan mijn studies en mijn (prille) wetenschappelijke carrière. Ik zou willen beginnen bij de kameraden die reeds het langst met mij opgescheept zitten. **Silke**, jij bent al zo lang mijn beste maatje dat ik wel met jou moet beginnen. Je bent er altijd voor mij geweest tijdens moeilijke momenten maar gelukkig worden die overheerst door de vele leuke dingen die we samen hebben meegemaakt. Ik hoop dat je voor altijd mijn maatje blijft en dat we nog vele feestjes kunnen bouwen samen. Ook nog bedankt om enkele keren mijn leven te redden toen ik door omstandigheden geen rijdende voertuigen meer zag aankomen, zonder jou(w reactievermogen) had ik hier letterlijk niet gestaan. **Jonas**, ik vind het uiteraard jammer dat je niet meer zo dichtbij woont, maar ben blij dat je content bent in Gent en dat je toch nog af en toe de weg naar Leuven vindt. **Sander**, nogmaals proficiat met uw aankoop, ik kijk al uit naar het eerste oogstfeest op uw boerderij. **Julien**, we zijn elkaar even uit het oog verloren, maar gelukkig is daar recent verandering in gekomen. Morgen geven we een kickass party in de sojo, like in the old days! **Ewoud**, blij dat ik steeds bij u kon komen chillen of met muziek bezig kon zijn, binnenkort zullen we zeker onze eerste "Dubsidizer meets General Fonz" opnemen! **Stefan**, ik hoop dat het plezant was in Oostenrijk, maar nu is het terug tijd om de Belgische feestjes en de tribunes van OHL onveilig te maken! **Brecht**, onze lasboyscout, ik ben super content dat wij als enigen zijn blijven supporteren voor OHL, ik hoop dat we onze vlag en sjaal nooit aan de haak zullen moeten hangen en dat we nog tientallen jaren lawijt mogen maken! **Cajo**, van feestkapitein tot echte family man, hopelijk word ik ook zo'n geweldige vader als jij. **Joeri**, ik zie u helaas veel te weinig, maar als ik u zie wordt het kot nog steeds met de grond gelijk gemaakt, zoals het hoort dus. **Jef**, ik ben blij dat je nu wél tevreden bent met je huidige job in Hoegaarden, spring zeker nog maar eens binnen na't werk! **Lars**, door omstandigheden zie ik ook jou veel te weinig, maar ik ben super trots dat je ondertussen aan het hoofd staat in de keuken van de Faculty

"Walking with a friend in the dark is better than walking alone in the light"
Helen Keller

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Beste vrienden, onze vriendschappen gaan al even mee en ik ben er zeker van dit die nooit zullen eindigen. Het spijt me als ik jullie de laatste jaren wat meer in de steek heb moeten laten, maar ik weet dat jullie me dit nooit kwalijk hebben genomen. Echte vrienden zijn namelijk diegenen waarbij het lijkt alsof het gisteren was, terwijl je elkaar eigenlijk al twee jaar niet gezien hebt, een gevoel dat ik bij ieder van jullie heb. Woorden kunnen eigenlijk niet beschrijven hoe nauw jullie mij aan het hart liggen dus ik zal het hierbij laten.

“One good thing about music, when it hits, you feel no pain”
Robert Nesta (Bob) Marley

Ten slotte wil ik dit uitgebreid dankwoord afsluiten met de familieleden te bedanken die mij al heel mijn leven steunen en aanmoedigen. **Tante Anne**, aan jou heb ik altijd alles kunnen vragen en jouw creatieve geest zorgt er steeds voor dat elk feest net dat tikkeltje extra heeft. Dit was dan ook duidelijk te zien op ons trouwfeest, maar ook op de andere talrijke familiefeestjes. Je bent een super tante waar ik veel aan heb en altijd op zal kunnen rekenen.

Seppe, mijn beste neef, we hebben samen al vele leuke tijden beleefd op de voetbal, op vakantie en op festivals. Ik hoop dan ook dat ik nog heel lang en heel vaak uw (zatte) "begeleider" mag zijn bij zulke aangelegenheden. Je bent ondertussen zelf aan je hogere studies begonnen en ben dan ook super fier dat je zonder problemen je eerste jaar bent doorgekomen. Ook in het rolstoelhockey gaat het steeds beter, ik zal je dan ook komen aanmoedigen waar en wanneer ik kan! **Ieke**, snotjonk, als kind trokken we al veel samen op en nadien in het middelbaar hebben we ook vele gekke avonturen meegemaakt. Ik ben dan ook heel blij dat we hier nu in Tienen een vervolg aan kunnen brijen en dat uw dochters veel slechte manieren aan mijne kleine kunnen aanleren. **Sofie**, het laatste familielid dat de Tienen-clan heeft vervoegd, ik hoop dat ik jou en Myrthe dan ook terug wat vaker zal zien en dat jullie ooit terug een OHL-abonnement zullen nemen. **Oma**, ik zou eigenlijk een apart boekje kunnen vullen met wat jij allemaal voor mij gedaan hebt en voor mij betekent. Al van sinds mijn geboorte heb ik zoveel nuttige dingen van je geleerd en meegekregen: leren tellen, leren rekenen, Leuvens dialect, ijsjesdecoratie in brand steken, het vakkundig wegjagen van jehova's getuigen en nog zoveel meer. Jij bent één van de personen die ervoor gezorgd heeft dat ik nu sta waar ik sta en dat ik ben wie ik nu ben en daar ben ik je eindeloos dankbaar voor. **Lies**, je bent dan wel een halve zus, toch ben je minstens even slim en prettig gestoord als uw broer. Ik vind het jammer dat we elkaar zo weinig zien en hoop dat daar snel verandering in zal komen als je voor de tweede keer tante wordt. Binnenkort zal jij ook afstuderen, je wordt vast en zeker een fantastische lerares. Weet dat ik je heel graag zie en dat je altijd welkom zal zijn bij ons. **Maaike**, je bent dan wel mijn hele zus, toch ben je minstens maar half zo lelijk als uw broer. Vroeger krabde je het liefst mijn armen en gezicht open en sloeg ik jou het liefst de kop in, gelukkig zijn die tijden veranderd en beseffen we beiden meer dan ooit dat we elkaar nodig hebben. Je bent een fantastische zus die het steeds opneemt voor haar familie. **Mike,** we kennen elkaar toch ook al even en ik ben blij voor u dat mijn zus het met u volhoudt. Vorig

"I don't know half of you half as well as I should like; and I like less than half of you half as well as you deserve."

Bilbo Baggins

jaar zorgden jullie ervoor dat ik de fierste peter ter wereld werd en ik beloof jullie dan ook dat ik altijd zal klaarstaan voor Lexie met raad en daad. Volgend jaar heeft ze er een speelkameraadje bij, al hoop ik dat die twee van in het begin overeen komen zonder al te veel ruzie.

Tussen het bedanken van mijn zusjes en mijn ouders moet ik ook mijn schoon(stief)ouders bedanken (anders mag ik misschien niet meer binnen). **Karine** (en **Benny**), **Wim** (en **Monica**) bedankt om mij van in het begin zo welkom te laten voelen, zelfs al zag ik er zo raar en ongewassen uit. Ook jullie hebben mij van bij het begin gesteund, ik ben dan ook heel blij dat jullie mijn schoonouders zijn. Ook mijn schoonzus **Chiara** mag ik niet vergeten aangezien ik haar door de jaren heen mocht pesten en steken geven, maar ook omdat we samen al goed gelachen hebben. Mijne kleine gaat twee superleuke meters hebben en daar ben ik enorm dankbaar voor.

Pa, jammer genoeg zag ik jou vroeger slechts om de twee weken en was je dus minder rechtstreeks betrokken bij mijn opvoeding. Toch heb ik veel van je geleerd en kon/kan ik altijd op jou rekenen, je zorgde ervoor dat ik nooit iets tekortkwam. Je hebt me steeds aangemoedigd en onvoorwaardelijk gesteund, ik weet maar al te goed hoe trots je op mij was/bent en dat is voor mij van onschatbare waarde. Binnen een half jaar wordt je voor de tweede keer opa en ik hoop dat je jouw waarden en normen ook aan je kleinkind zal aanleren zoals je bij mij hebt gedaan. **Mams**, als er iemand is die mijn liefde voor wetenschap meteen zag en voedde dan was jij het wel. Jarenlang gingen we samen naar de bib en kwam ik telkens terug met enkele kilo's aan encyclopedieën en wetenschapsboeken. Toen ik een chemiedoos vroeg aan de sint, kreeg ik die, ook al was ik nog enkele jaren te jong daarvoor (bij deze nogmaals sorry voor het in brand steken van de tafel en andere experiment-gerelateerde incidenten). Verder heb je mij en Maai altijd op de eerste plaats gezet, nu nog steeds trouwens. Wat wij ook aan jou vragen, niets is jou te veel. Ik ben dan ook super content dat mijne kleine zo'n fantastische oma gaat hebben!

Ik ben nu bijna aan het einde van mijn dankwoord gekomen en ik zou graag eindigen met de belangrijkste vrouw in mijn leven. **Yentel,** lieve schat, jarenlang heb je tot vervelens toe mijn gezeur moeten aanhoren over hoe druk het was en hoeveel tijd ik tekortkwam. Toch was jij

"Family is not an important thing.
It's everything."
Michael J fox

de enige die me weer rustig kreeg wanneer ik weer eens aan het stressen en vloeken was. We zijn nu tien jaar samen en jij maakte in die tijd van deze ongewassen, langharige, werkschuwe vreemde vogel een (semi-)deftige, gedoctoreerde vreemde vogel. Ik ben er heilig van overtuigd dat ik zonder jou hier vandaag niet had gestaan. Binnen een half jaar zal ons kindje op de wereld komen, een dag waar ik enorm naar uitkijk. Het is allemaal heel spannend, maar ik weet dat jij een fantastische mama zal zijn. Bovendien kunnen wij als team de hele wereld aan en dat zal ook deze keer niet anders zijn. Ik hou enorm veel van je en zal dat ook altijd blijven doen.

"If she's amazing, she won't be easy. If she's easy, she won't be amazing. If she's worth it, you won't give up. If you give up, you're not worthy. ... Truth is, everybody is going to hurt you; you just gotta find the ones worth suffering for."

Robert Nesta (Bob) Marley

CURRICULUM VITAE

Personal information

Name: Vanhove

First name: Wiebe

Date of birth: October 13, 1988

Place of birth: Leuven

Gender: Male

Nationality: Belgian

Address: Vissenakenstraat 52, 3300 Tienen

E-mail: wiebe.vanhove@kuleuven.be

Education

2013-current: PhD in Biomedical Sciences: *Molecular characterization of pathophysiologic pathways in IBD and its therapeutic potential*, KU Leuven, Leuven, Belgium

2010-2013: Master in Biology, KU Leuven, Leuven, Belgium (Cum laude)

2007-2010: Professional Bachelor of Bio-Medical Laboratory Technology (Option in Pharmaceutical and Biological Laboratory Technology), KH Leuven (UCLL), Leuven, Belgium (Magna cum laude)

2000-2006: High School: Sciences – Modern Languages, KA2 (Ring), Leuven, Belgium

Professional experience

PhD student in biomedical sciences (2013-current):
Translational Research in GastroIntestinal Disorders (TARGID), Department of Clinical and Experimental Medicine, KU Leuven, Leuven, Belgium - Promoter: Prof. Dr. Séverine Vermeire; Co-promoters: Dr. Ingrid Arijs and Dr. Kris Nys

Master Thesis in Biology (2012-2013):
Laboratory of Animal Physiology and Neurobiology, Department of Biology, KU Leuven, Leuven, Belgium - Promoter: Prof. Dr. Lieve Moons

Summer job lab technician (2011 & 2012):
Translational Research in GastroIntestinal Disorders (TARGID), Department of Clinical and Experimental Medicine, KU Leuven, Leuven, Belgium

Summer job lab technician (2010):
Gene Expression Unit, Department of Cellular and Molecular Medicine, KU Leuven

Bachelor thesis (2009-2010):

Gene Expression Unit, Department of Cellular and Molecular Medicine, KU Leuven – Promoter: Prof. Dr. Frans Schuit

Scientific skills and additional education

Microsoft Office (Word, Excel, Powerpoint), R statistics, ImageJ, GraphPad Prism, PLINK

Laboratory Animal Science Module 1 (2009) and Module 2 (2014)

Statistics course (2013)

Scientific writing course (2013)

Presentation skills course (2014)

UEG Basic Science course (Amsterdam, 2015)

Charles River and Jackson laboratories seminar (2016)

Scientific meetings and international conferences

Belgian Week of Gastroenterology (BWGE) – La Hulpe, Belgium, February 2014 (Oral poster)

Digestive Disease Week (DDW) – Chicago, IL, USA, May 2014 (Poster)

BWGE – Brussels, Belgium, February 2015 (Oral)

European Crohn's and Colitis (ECCO) Conference – Barcelona, Spain, February 2015 (Poster)

Ghent Gut Inflammation Group Meeting – Ghent, Belgium, March 2015 (Passive)

DDW – Washington, DC, USA, May 2015 (Passive)

BWGE – Brussels, Belgium, February 2016 (Oral)

ECCO – Amsterdam, Netherlands, March 2016 (Poster)

DDW – San Diego, CA, USA, May 2016 (Poster)

BWGE – Antwerp, Belgium, February 2017 (Oral)

ECCO – Barcelona, Spain, February 2017 (Oral & Poster)

DDW - Chicago, IL, USA, May 2017 (Poster)

SCIENTIFIC COMMUNICATIONS

Peer reviewed publications

De Preter V, Arijs I, Windey K, **Vanhove W**, Vermeire S, Schuit F, Rutgeerts P, Verbeke K. Impaired butyrate oxidation in ulcerative colitis is due to decreased butyrate uptake and a defect in the oxidation pathway. Inflamm Bowel Dis. 2012 Jun;18(6):1127-36.

De Preter V, Arijs I, Windey K, **Vanhove W**, Vermeire S, Schuit F, Rutgeerts P, Verbeke K. Decreased mucosal sulfide detoxification is related to an impaired butyrate oxidation in ulcerative colitis. Inflamm Bowel Dis. 2012 Dec 18(12):2371-80.

Arijs I, **Vanhove W**, Rutgeerts P, Schuit F, Verbeke k, De Preter V. Decreased mucosal sulfide detoxification capacity in Crohn's disease patients. Inflamm Bowel Dis. 2013 Apr 19(5):E70-2

Van der Goten J, **Vanhove W**, Lemaire K, Van Lommel L, Machiels K, Wollants WJ, De Preter V, De Hertogh G, Ferrante M, Van Assche G, Rutgeerts P, Schuit F, Vermeire S, Arijs I. Integrated miRNA and mRNA expression profiling in inflamed colon of patients with ulcerative colitis. PLoS One. 2014 Dec 9(12):e116117.

Boesmans L, Ramakers M, Arijs I, Windey K, **Vanhove W**, Schuit F, Rutgeerts P, Verbeke K, De Preter V. Inflammation-Induced Downregulation of Butyrate Uptake and Oxidation Is Not Caused by a Reduced Gene Expression. J Cell Physiol. 2015 Feb;230(2):418-26

Vanhove W, Peeters PM, Staelens D, Schraenen A, Van der Goten J, Cleynen I, De Schepper S, Van Lommel L, Reynaert NL, Schuit F, Van Assche G, Ferrante M, De Hertogh G, Wouters EF, Rutgeerts P, Vermeire S, Nys K, Arijs I. Strong Upregulation of AIM2 and IFI16 Inflammasomes in the Mucosa of Patients with Active Inflammatory Bowel Disease. Inflamm Bowel Dis. 2015 Nov 21(11):2673-82.

Vanhove W, Nys K, Vermeire S. Therapeutic innovations in inflammatory bowel diseases. Clin Pharmacol Ther 2016 Jan;99(1):49-58

Vanhove W, Peeters PM, Cleynen I, Ferrante M, Vermeire S, Arijs I. Absent in melanoma 2 (AIM2) in the intestine: diverging actions with converging consequences? Inflammasome 2016, 3(1), pp. 1-9

Arijs I, De Hertogh G, Lemmens B, Van Lommel L, de Bruyn M, **Vanhove W**, Cleynen I, Machiels K, Ferrante M, Schuit F, Van Assche G, Rutgeerts P, Vermeire S. Effect of vedolizumab (anti-α4β7-integrin) therapy on histological healing and mucosal gene expression in patients with UC. Gut. 2016 Oct. [Epub ahead of print]

Vanhove W, Nys K, Arijs I, Cleynen I, De Schepper S, Van Assche G, Ferrante M, Vermeire S. Biopsy-derived intestinal epithelial cell cultures for pathway based stratification of IBD patients [Accepted for publication in JCC]

Publications submitted or in preparation

Vanhove W, Nys K, Merciris D, Laukens D, Vayssière B, Auberval M, Ongenaert M, De Vriendt V, Montjardet A, Borgonovi M, Lepescheux L, Dupont S, Clément-Lacroix P, De Vos S, De Vos M, Brys R, Vermeire S, Galien R. Selective inhibition of Janus kinase 1 (JAK1) with filgotinib reverses pathogenic processes in preclinical models for IBD. [This manuscript is in preparation for submission in Gastroenterology]

Presentations at scientific meetings

Vanhove W, Staelens D, Peeters P, Van der Goten J, Wouters EF, Van Assche G, Ferrante M, Vermeire S, Rutgeerts P, Nys K, Arijs I. Different inflammasome subtypes are activated in human inflammatory bowel diseases. *Poster presentation* at the XXVIth Belgian Week of Gastroenterology, Dolce - La Hulpe, February 12-15, 2014.

Vanhove W, Staelens D, Peeters P, Van der Goten J, De Schepper S, Cleynen I, Wouters EFM, Van Assche G, Ferrante M, Vermeire S, Rutgeerts P, Nys K, Arijs I. Strong mucosal upregulation of AIM2 and IFI16 inflammasomes in patients with active inflammatory bowel disease. *Poster presentation* at the Digestive Disease Week (DDW), Chicago, Illonois, USA, May 3 - 6, 2014.

Vanhove W, De Schepper S, Staelens D, Arijs I, Van Assche G, Ferrante M, Vermeire S, Nys K. Patient-derived colonic epithelial cultures as a valuable tool for personalized medicine. *Poster presentation* at the 10th Congress of the European Crohn's and Colitis Organisation (ECCO), Barcelona, Spain, February 18 – 21, 2015

Vanhove W, De Schepper S, Staelens D, Arijs I, Van Assche G, Ferrante M, Vermeire S, Nys K. Culturing patient derived intestinal epithelium as a valuable tool for personalized medicine. *Oral presentation* at the XXVIIth Belgian Week of Gastroenterology, Square - Brussels, February 25-28, 2015

Vanhove W, Nys K, Arijs I, Cleynen I, Van Assche G, Ferrante M, Vermeire S. Functional translation of IBD-associated genetic variation in patient-derived intestinal epithelial cells. *Oral presentation* at the XXVIIIth Belgian Week of Gastroenterology, Square - Brussels, February 18 - 20, 2016

Vanhove W, Nys K, Arijs I, Cleynen I, Van Assche G, Ferrante M, Vermeire S. Functional translation of IBD-associated genetic variation in patient-derived intestinal epithelial cells. *Poster presentation* at the 11th Congress of the European Crohn's and Colitis Organisation (ECCO), Amsterdam, Netherlands, March 16 – 19, 2016

Vanhove W, Nys K, Arijs I, Cleynen I, Van Assche G, Ferrante M, Vermeire S. Functional translation of IBD-associated genetic variation in patient-derived intestinal epithelial cells. *Poster presentation* at the Digestive Disease Week (DDW), San Diego, California, USA, May 21 - 24 2016

Vanhove W, Nys K, Arijs I, Cleynen I, de Bruyn M, Korf H, Ferrante M, Van Assche G, Vermeire S. Defects in ER stress and autophagy genes translate into increased functional ER stress levels in patients with inflammatory bowel disease. *Oral presentation* at the XXIXth Belgian Week of Gastroenterology, Antwerp, February 9 - 11, 2016

Vanhove W, Nys K, Arijs I, Cleynen I, de Bruyn M, Korf H, Ferrante M, Van Assche G, Vermeire S. The genetic risk in ER stress and autophagy translates into quantifiable epithelial ER stress levels in IBD patients. *Oral and poster presentation* at the 12th Congress of ECCO. Barcelona, Spain, February 15-18, 2017

Vanhove W, Nys K, Arijs I, Cleynen I, de Bruyn M, Korf H, Ferrante M, Van Assche G, Vermeire S. Genetic defects in ER stress and autophagy translate into increased functional ER stress levels in patients with inflammatory bowel disease *Poster presentation* at the Digestive Disease Week (DDW), Chicago, Illinois, USA, May 6-9